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Physiological and molecular basis determining inter-individual growth rate differences in spat of the mussel *Mytilus galloprovincialis* reared under different nutritional and temperature environments

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Introducción

1- Presentación

El crecimiento es el proceso mediante el cual se produce el aumento de tejido somático y/o reproductivo y se considera un indicador del estatus fisiológico general de los organismos. El crecimiento es la consecuencia de la existencia de un balance energético neto positivo entre los flujos de ingreso y pérdida de energía a través del individuo. Las condiciones ambientales influyen de manera determinante sobre el resultado del balance energético de los organismos, y por consiguiente sobre su crecimiento, ya que afectan tanto a la posibilidad de incorporar energía en forma de alimento, como a los costes metabólicos del conjunto de actividades requeridas al objeto de garantizar la supervivencia y los procesos propios de crecimiento y reproducción. Las oscilaciones de las condiciones ambientales son especialmente relevantes en el caso de los bivalvos, ya que éstos son organismos sésiles que habitan el medio litoral, un entorno especialmente variable sometido a intensas oscilaciones de sus parámetros físico-químicos. Cabe destacar que las variables ambientales que más efecto tienen sobre el crecimiento en los moluscos bivalvos son la temperatura y la disponibilidad de alimento, tanto en términos de cantidad como de calidad. El efecto de ambos factores sobre el crecimiento y los componentes fisiológicos que determinan el balance energético han sido profusamente estudiadas en los bivalvos, y se ha demostrado que estos organismos poseen una gran variedad de mecanismos de compensación fisiológica que les permite preservar la actividad biológica y el crecimiento en un entorno especialmente variable como el litoral (Thompson and Bayne 1974; Winter 1978; Kiorboe et al. 1980; Okumus and Stirling 1994; Navarro et al. 1994; Pernet et al. 2007). Precisamente la plasticidad fisiológica puede considerarse una de las principales razones por las cuales los bivalvos presentan una amplia distribución y son capaces de habitar los ecosistemas intermareales, que se caracterizan por estar sometidos a continuas fluctuaciones ambientales.

La variabilidad en el crecimiento de los moluscos bivalvos no sólo viene determinada por variables exógenas. Existen numerosos trabajos que muestran la existencia de una alta variabilidad en las tasas de crecimiento de individuos pertenecientes a una misma población (tanto en poblaciones naturales como procedentes

de plantas de cultivo) mantenidos bajo idénticas condiciones ambientales y/o experimentales (Bayne 1999a, 1999b; Toro et al. 2004; Tamayo et al. 2011) que indican la existencia de diferencias de origen endógeno (genético o epigenético) en la capacidad de adquirir-procesar alimento y/o en los costes energéticos de los procesos metabólicos que pueden generar notables diferencias de talla o tamaño entre individuos. La existencia de diferencias inter-individuales tan marcadas constituye un fenómeno biológico de gran interés para la acuicultura ya que ofrece un amplio margen para la implementación de procesos de mejora de la productividad. Son numerosos los estudios que en los últimos tiempos se han centrado en la caracterización de las bases fisiológicas y moleculares responsables de las diferencias inter-individuales en tasa de crecimiento. Al igual que con las variables exógenas, analizaré más a fondo los resultados obtenidos por diferentes autores más adelante, pero al objeto de contextualizar el trabajo, sí me parece oportuno indicar aquí que la información disponible sobre los condicionantes moleculares del crecimiento en bivalvos es muy escasa en comparación con la que se tiene para otros grupos animales, probablemente debido al lento desarrollo de la investigación genómica en estos organismos. La existencia de diferencias tan pronunciadas en la tasa de crecimiento entre individuos de la misma población evidencia que la capacidad de explotar los recursos tróficos y/o de acometer respuestas de compensación fisiológica frente a las oscilaciones de las condiciones ambientales presenta un alto grado de variabilidad inter-individual. En el ámbito de la fisiología energética, existe cierta discrepancia sobre la contribución que los distintos parámetros fisiológicos ejercen sobre las diferencias interindividuales en la tasa de crecimiento: mientras algunos autores defienden que las diferencias inter-individuales en tasa de crecimiento se derivan de diferencias en la capacidad de los procesos de adquisición y absorción del alimento, otros autores sostienen que las diferencias fundamentales son de carácter metabólico.

Recientemente, nuestro grupo de investigación ha publicado un conjunto de trabajos (Tamayo et al. 2011, 2013, 2014, 2016) que apuntan a la idea de que las condiciones ambientales (en concreto las condiciones nutricionales) reinantes durante el periodo de crecimiento y diferenciación en tamaño determinarían las diferencias en rasgos fisiológicos (o fenotípicos) entre individuos segregados por su tasa de crecimiento. Dicho de otra manera, los procesos fisiológicos responsables de la diferenciación en tamaño entre individuos de una población podrían no ser los mismos

en todas las circunstancias, sino que podrían estar determinados por las condiciones ambientales bajo las que se desarrollan las poblaciones. Así, podría considerarse que un rasgo que podría resultar ventajoso para el mantenimiento de altas tasas de crecimiento bajo unas condiciones ambientales determinadas podría, sin embargo, no favorecer el crecimiento bajo otras condiciones ambientales distintas. Es decir, un fenotipo determinado podría presentar elevadas tasas de crecimiento en un ambiente concreto, pero no resultar tan exitoso en otro.

El objetivo de esta tesis es analizar la existencia de diferencias inter-individuales en la tasa de crecimiento de origen endógeno en juveniles del mejillón *Mytilus galloprovincialis* mantenidos en el laboratorio bajo condiciones controladas que simulan diferentes escenarios de disponibilidad de alimento y temperatura, y establecer si las diferencias en los perfiles fisiológicos entre individuos de alta y baja tasa de crecimiento (F: fast growers y S: slow growers, respectivamente) persisten o difieren en los diferentes escenarios. Con este objeto se han realizado experimentos en los que una gran cantidad de semillas de mejillón recogidas de un roquedo intermareal, y de tamaño homogéneo, se mantenían en el laboratorio bajo condiciones (estrictamente controladas) homogéneas de alimentación y temperatura durante el tiempo requerido (de 3 a 11 meses dependiendo de las condiciones) para la aparición de diferencias en talla que permitían seleccionar grupos de alta y baja tasa de crecimiento (F y S). En esta tesis se han considerado 5 escenarios ambientales diferentes a fin de analizar los efectos de la disponibilidad de alimento (capítulos 1 y 2) y otros dos para establecer el efecto de la temperatura (capítulo 3) en la capacidad para segregar grupos F y S. La diferencia en los rasgos fisiológicos de los individuos F y S en cada uno de los siete escenarios se analizó mediante la determinación experimental del conjunto de parámetros que componen el balance energético.

Un objetivo complementario de este trabajo es tratar de establecer las bases genéticas que subyacen en las diferencias interindividuales en tasa de crecimiento en el mejillón *Mytilus galloprovincialis.* A tal efecto se han efectuado análisis comparativos del transcriptoma de los grupos F y S mediante la utilización de microarrays de expresión (capítulo 4). Las muestras en las que se analizó el transcriptoma correspondían a individuos utilizados en los experimentos de fisiología, con lo que la interpretación combinada de ambas aproximaciones ofrece una visión holística del funcionamiento del organismo.

2- Balance energético y relaciones funcionales entre parámetros fisiológicos y variables ambientales

La aplicación de los principios de la termodinámica al estudio de los intercambios energéticos de los organismos con su ambiente viene recogida en la ecuación del balance energético:

$$I= R+F+U+P$$

donde I representa la energía ingerida a través del consumo de alimentos, R es la energía disipada en forma de calor como consecuencia de la transformación energética en los procesos metabólicos, F es la energía devuelta al entorno en forma de heces, U es la energía devuelta al entorno en forma de compuestos orgánicos (básicamente restos nitrogenados) excretados en la orina y P representa la energía retenida en el organismo. Así, esta última representa la energía que queda disponible para el crecimiento (P, producción) y vendría dada como la diferencia entre ingreso y pérdida de energía:

$$P= I-(F+U+R)$$

Los componentes de entrada y pérdida de energía indicados en la ecuación del balance energético son mensurables, y su determinación experimental, a nivel de individuo mediante técnicas de fisiología energética permite estimar la energía retenida por unidad de tiempo por el individuo. Dicha estima recibe el nombre de alcance energético para el crecimiento (Warren and Davis, 1967), aunque comúnmente se utiliza el término inglés Scope for growth (SFG). El SFG es una medida que proporciona una estimación razonable del potencial de crecimiento del individuo en las condiciones ambientales o experimentales bajo las cuales se realiza la determinación de los parámetros del balance energético. La determinación experimental de la tasa real de crecimiento de un individuo, como diferencia de talla o peso en un período de tiempo determinado, exige diseños experimentales extensos, temporalmente hablando. La tasa de crecimiento es una medida que integra los posibles efectos de las modificaciones ambientales y/o de estado fisiológico del organismo que tienen lugar durante todo el periodo de tiempo que comprende la medida. El SFG, sin embargo, constituye una estima instantánea del potencial crecimiento del organismo, y por consiguiente no es una medida integradora. Pero la relativa rapidez con la que es posible cuantificar el SFG, permite analizar de manera relativamente cómoda y exhaustiva el efecto que sobre el crecimiento tienen las variables ambientales y endógenas que afectan a la actividad y

el metabolismo de los organismos. La investigación sobre los efectos de las variables tanto exógenas (disponibilidad de alimento, temperatura, salinidad) como endógenas sobre el crecimiento en bivalvos se ha beneficiado enormemente de este tipo de metodología y, por consiguiente, existe abundante literatura sobre estas cuestiones basadas en la utilización de metodologías de fisiología energética. Puesto que el presente trabajo está principalmente basado en la comparación de los componentes fisiológicos del balance energético entre individuos de alta y baja tasa de crecimiento seleccionados bajo diferentes condiciones de disponibilidad de alimento y temperatura, se ha considerado oportuno presentar aquí una revisión bibliográfica de los efectos conocidos de estas dos variables sobre el balance energético y el crecimiento en bivalvos. Adicionalmente se describirán la dependencia que ciertos parámetros fisiológicos tienen entre sí, a fin de facilitar la comprensión de los resultados de los experimentos de fisiología que se expondrán en los distintos capítulos de este trabajo de tesis doctoral.

2.1 Procesos de adquisición de energía

2.1.1 Filtración del agua y adquisición de las partículas alimenticias

Aspectos morfo-funcionales de los órganos de adquisición de alimento. Los moluscos bivalvos son organismos filtradores. La adquisición del alimento corre a cargo de la branquia y de los palpos labiales. La branquia o ctenidio de los moluscos bivalvos se sitúa en la cavidad paleal y consta de 4 pares de demibranquias compuestas por lamelas, que a su vez están constituidas por filamentos que presentan abundantes zonas ciliadas. El movimiento de los cilios latero-frontales (Riisgard et al. 1996; Ward et al. 1998) de los filamentos genera una corriente de agua que discurre a través de la branquia. El agua bombeada a través de la apertura (o sifón) inhalante ingresa en la cavidad paleal, atraviesa los espacios inter-filamentares de la branquia, y es expelida a través de la apertura (o sifón) exhalante. Los cilios branquiales actúan como un tamiz, filtrando el agua y reteniendo las partículas suspendidas en ella. Cabe destacar que la eficiencia de retención de las partículas en la branquia depende en gran medida del tamaño de las partículas. Obviando las diferencias inter-especificas, se acepta que, en general, la eficiencia de retención de la branquia se aproxima al 100% cuando las partículas superan los 3 µm de diámetro, pero se reduce a valores próximos al 50% con

partículas de diámetros inferiores (Møhlenberg and Riisgård 1978; Stuart and Klumpp 1984; Riisgård 1988; De Villiers and Allanson 1988). No obstante, la eficiencia de retención también parece depender de otras características de las partículas. Por ejemplo, en experimentos realizados con individuos de ostra *Ostrea edulis* (Shumway et al. 1985), mejillón *Mytilus edulis* (Newell et al. 1989) y pectínidos *Placopecten magellanicus* (Lesses et al. 1991), se ha constatado la existencia de diferencias substanciales en la eficiencia de retención de partículas que, aun teniendo el mismo tamaño, presentaban una distinta composición química. Para un análisis más profundo sobre los factores que afectan a la eficiencia de retención recomendamos la revisión de Ward y Shumway (2004).

Las partículas retenidas por los cilios frontales son transportadas hacía los tractos ciliados dorsal y ventral de la branquia, y una vez allí son embebidas en moco y conducidas hacia la parte anterior de la branquia donde se sitúa la boca y los palpos labiales. Este órgano peri-bocal (palpos labiales) ejerce una función fundamental en la alimentación y la regulación de la tasa de ingestión en los bivalvos: su cometido es recoger las partículas y ejercer un proceso de selección pre-ingestiva de las mismas. Las partículas aceptadas para su ingestión son transportadas a la boca, mientras que las partículas rechazadas son devueltas al entorno embebidas en una secreción mucosa, formando biodepósitos conocidos con el nombre de pseudoheces. El rechazo de una fracción del alimento filtrado en forma de pseudoheces constituye un mecanismo de limitación, y por lo tanto de regulación, de la tasa de ingestión (Widdows et al. 1979; Bayne et al. 1989; Navarro et al. 1992). Por otra parte, se ha demostrado que la selección pre-ingestiva realizada por los palpos labiales implica el rechazo preferente de materia inorgánica y la ingestión preferente de materia orgánica, lo que da lugar al enriquecimiento de la calidad o proporción orgánica de la materia ingerida respecto a la filtrada (Kiorboe and Mohlenberg 1981; Koehn and Shumway 1982; Newell and Jordan 1983; Iglesias et al. 1992; Urrutia et al. 1996, 2001). Además, la capacidad selectiva de los palpos no se restringe al discernimiento entre materia orgánica e inorgánica. Se ha observado que los bivalvos pueden diferenciar entre partículas orgánicas de distinta naturaleza, por ejemplo, seleccionando preferentemente células fitoplanctónicas vivas frente a muertas (Cognie et al. 2003; Beninger et al. 2004), o fitoplancton frente a detrito (Ward et al. 1997; 1998; Milke and Ward, 2003).

Parámetros fisiológicos de los órganos de adquisición de alimento. El análisis de la actividad desarrollada por la branquia en la adquisición de partículas de alimento se lleva a cabo mediante la determinación de una serie de parámetros que se presentan a continuación. La *tasa de aclaramiento (CR: l/h)* es el parámetro que comúnmente se utiliza para describir la velocidad con la que los bivalvos filtran el agua para adquirir las partículas de alimento. La tasa de aclaramiento se define como el volumen de agua totalmente aclarado de partículas en suspensión por unidad de tiempo. La *tasa de filtración (FR: mg/h)* representa la masa total de las partículas retenidas o filtradas en la branquia por unidad de tiempo, y se obtiene como el producto de la tasa de aclaramiento y la concentración de materia en suspensión, siempre y cuando la eficiencia de retención del conjunto de partículas en la suspensión alcance el valor de 100%. La *tasa de rechazo (RR: mg/h)* representa la masa total de partículas rechazadas en forma de pseudoheces por unidad de tiempo. La cantidad de materia ingerida por unidad de tiempo o *tasa de ingestión (IR: mg/h)* se obtiene por diferencia entre tasa de filtración y tasa de rechazo. En ausencia de rechazo de alimento en forma de pseudoheces, las tasas de filtración e ingestión son equivalentes.

Efectos de las variables ambientales sobre la adquisición y selección preingestiva del alimento. La actividad alimenticia de los bivalvos ha sido analizada con profusión a lo largo de las seis últimas décadas y, por consiguiente, existe una vasta cantidad de información relativa a los aspectos funcionales de los procesos de filtración, selección pre-ingestiva, rechazo e ingestión del alimento, así como sobre el efecto que las variables ambientales ejercen sobre dichos procesos. En general, se ha observado que los procesos pre-ingestivos de los bivalvos presentan una enorme plasticidad fisiológica que permite a estos organismos adecuar las tasas de aclaramiento y rechazo, así como de la selección pre-ingestiva, a las continuas fluctuaciones tanto de las características del alimento disponible como de la temperatura del agua. Las características de la materia particulada en suspensión, fundamentalmente la concentración y la calidad o proporción de materia orgánica en la misma, ejercen un efecto decisivo sobre la tasa de aclaramiento de los bivalvos. En numerosas ocasiones, tanto en trabajos realizados en condiciones de laboratorio como en el entorno natural, se ha observado la existencia de una relación inversa entre concentración de partículas y tasa de aclaramiento (Winter 1973, 1978; Foster and Smith 1975; Bayne and Newell 1983; Galimany et al. 2011; Tamayo et al. 2016; Kang et al. 2016). Esta reducción en la

tasa de aclaramiento ha sido interpretada como un mecanismo de regulación de la tasa de ingestión, cuya principal virtud sería la de mantener el tiempo de tránsito del alimento en valores adecuados para el mantenimiento de altos valores de eficiencia de absorción del alimento. El efecto de la ración o concentración de partículas sobre la tasa de aclaramiento depende en gran medida de la calidad o proporción de materia orgánica en la dieta. Navarro et al. (1994) analizaron la variación de las tasas de aclaramiento e ingestión en el berberecho *Cerastoderma edule* sometido a cambios en la calidad y concentración del alimento y observaron que la reducción de la tasa de aclaramiento con el incremento de la ración era tanto más acusada cuanto mayor era la calidad de la dieta. En un trabajo posterior, Navarro et al. (1996) determinaron la tasa de aclaramiento de mejillones *Mytilus galloprovincialis* alimentados con raciones similares de 5 dietas que diferían entre sí en el contenido orgánico de la dieta (de 16 a 91%) y observaron que la tasa de aclaramiento aumentaba cuanto menor era porcentaje orgánico en la dieta hasta un valor de aproximadamente 30%, si bien la reducción en el porcentaje orgánico de la dieta a valores menores del 30% (de 30 a 16%) daba lugar al efecto contrario y provocaba la reducción de la tasa de aclaramiento. La interacción de las variables cantidad y calidad de alimento sobre la tasa de aclaramiento tiene su explicación, según Navarro y colaboradores, en el proceso de selección y rechazo pre-ingestivo del alimento llevado a cabo por los palpos labiales. La eficiencia con las que los palpos labiales rechazan el material inorgánico es altamente dependiente de la calidad del alimento en suspensión (Iglesias et al.1992; Urrutia et al.1996; Hawkins et al.1996): La eficiencia neta de selección presenta valores máximos en torno a contenidos orgánicos del 40%. El incremento del contenido orgánico a valores superiores al 40% provoca la reducción de la eficiencia de selección debido, probablemente, a una limitación en el proceso de selección derivada del descenso en la proporción de partículas inorgánicas en la suspensión (Ward and Shumway 2004). La reducción del contenido orgánico del alimento a valores inferiores al 40% también provoca la reducción de la eficiencia neta de selección, fundamentalmente debido al componente de pérdida de materia orgánica endógena que representa la secreción mucosa (Urrutia et al. 2001). A calidades altas, es decir cuando el contenido orgánico de la dieta es alto, la eficiencia de selección pre-ingestiva de partículas es reducida, de modo que la regulación de la tasa de ingestión se lleva a cabo recurriendo a la modulación de la tasa de aclaramiento (Widdows et al. 1979; Bayne et al. 1989), sin embargo, a calidades bajas, el mantenimiento de la tasa de aclaramiento respondería a los beneficios que se derivan de la posibilidad de ejercer un

proceso de selección de partículas que permite incrementar la proporción de materia orgánica en la ingesta y facilitar, en consecuencia, el subsiguiente proceso de digestión y absorción del alimento.

Como es habitual en organismos poiquilotermos como los bivalvos, el metabolismo y la actividad biológica presentan una elevada dependencia térmica. En general, en el rango de tolerancia térmica, la tasa de aclaramiento aumenta con la temperatura hasta alcanzar un valor máximo, a partir del cual desciende rápidamente (Schulte 1975; Newell et al. 1977; Jorgensen et al. 1990; Haure et al. 1998; Ezgeta Balic et al. 2011). La temperatura a la que dicho declive en la tasa de aclaramiento tiene lugar determina el límite superior del rango de tolerancia térmica del organismo, el cual varía entre especies (Portner 2001, 2002). Algunos autores han sugerido que los bivalvos poseen cierta capacidad de compensación inmediata de los efectos térmicos sobre la actividad filtradora puesto que en algunas especies se ha observado la existencia de ciertos rangos de termo-independencia de la tasa de aclaramiento (Schulte 1975; Newell et al.1977; Buxton et al.1981; Sara et al. 2008). También se ha observado que los bivalvos pueden presentar cierta capacidad para elaborar respuestas compensatorias crónicas (aclimatación) de la tasa de aclaramiento a los cambios de temperatura (Widdows, 1973) mediante la regulación de la composición lipídica (adaptación homeoviscosa) de las membranas de las células branquiales (Pernet et al. 2007; Parent et al. 2008).

2.1.2. Digestión y Absorción del alimento

Una vez ingeridas las partículas de alimento, acceden al estómago a través de un corto esófago. Las partículas alimenticias incorporadas al estómago son trituradas por la acción rotatoria del estilo cristalino. El estilo cristalino es una secreción hialina cilíndrica y semi-sólida, constituida por una matriz proteica en la que se integran distintos enzimas digestivos. La acción rotatoria del estilo cristalino se lleva a cabo gracias a los cilios de las células que componen el saco del estilo. Está acción rotatoria resulta crucial en la digestión de los bivalvos, ya que por un lado produce la digestión mecánica del alimento, y por otro, se liberan las enzimas encargadas de la digestión preliminar del alimento. El estómago de los bivalvos está comunicado con la glándula digestiva donde se produce la digestión intracelular, y con el intestino, donde se dirige

el material para su posterior egestión. La glándula digestiva está formada por túbulos ciegos que se comunican con el estómago mediante una serie de aberturas o *caecas*. Los túbulos glandulares están recubiertos por células digestivas, encargadas de la digestión intracelular y posterior absorción de las partículas alimenticias, y células basófilas que secretan enzimas digestivas que, incorporadas a las partículas de alimento, cooperan en la digestión intracelular del mismo en el interior del sistema lisosómico de las células digestivas. La asignación de partículas bien al intestino o a los divertículos de la glándula digestiva está sujeta a un proceso de selección (selección digestiva) previo en las abundantes regiones ciliadas del estómago. Una serie de trabajos pioneros en la materia establecieron que la selección digestiva opera por mecanismos de discriminación por tamaño (Owen 1974; Purchon, 1978): las partículas pequeñas pueden ingresar a los túbulos de la glándula digestiva para dar continuidad al proceso de digestión de las mismos, mientras que las partículas grandes, son redirigidas al intestino. La serie de trabajos de Brillant y MacDonald (2000, 2002 y 2003). utilizando partículas de poliestireno y diferentes partículas orgánicas para la alimentación de *Placopecten magellanicus*, concluyeron que la selección que tiene lugar en los zonas ciliadas del estómago probablemente combina la selección en base al tamaño de partícula con cierto reconocimiento a nivel físico-químico de la superficie de las partículas, dando como resultado la incorporación preferencial de materia orgánico a la glándula digestiva.

La hidrólisis de las macromoléculas orgánicas a sus unidades estructurales básicas (monómeros) en el interior de los lisosomas de las células digestivas permite la posterior absorción del alimento. En los bivalvos, el grueso de la absorción de alimento tiene lugar en el epitelio de la glándula digestiva mientras que el intestino queda relegado a cumplir poco más que una función de empaquetamiento de las heces. La tasa de absorción de materia orgánica (AR: mg/h) es el parámetro que representa la absorción neta de alimento en el digestivo, mientras que la eficacia relativa con la que el sistema digestivo obtiene energía a partir del alimento ingerido viene determinada por la eficiencia de absorción (AE: %), parámetro que se define como la proporción (porcentaje) de materia orgánica ingerida que es finalmente absorbida. Pues bien, como se describe a continuación, puede considerarse que hay al menos tres variables fisiológicas de las que depende la eficiencia de absorción: a) la capacidad total de los órganos para albergar alimento (capacidad digestiva: mg), b) la actividad del conjunto de enzimas digestivas que participan en la hidrólisis y absorción de las partículas

(Actividad enzimática: mg producto/h mg proteína) y c) el tiempo de residencia del alimento en el órgano digestivo (tiempo de paso: h).

Capacidad digestiva. La capacidad digestiva se define como el volumen o masa total de alimento albergado en el tubo digestivo y generalmente su valor se estima a partir de la cuantificación de la materia evacuada en forma de heces por un individuo durante un periodo de ayuno forzado. Se acepta generalmente que este parámetro representa la capacidad volumétrica que posee el sistema digestivo para albergar alimento, capacidad que no representa una mera magnitud física, sino que está afectada también por la acción de los enzimas digestivos, que provocan la hidrólisis y la consiguiente compactación de las partículas ingeridas. Precisamente por ello, no es de extrañar que se haya observado que los bivalvos puedan modificar la capacidad digestiva en respuesta a las variaciones de las características de la dieta (Bayne et al. 1987, 1989; Navarro et al. 1992, 1994; Navarro e Iglesias 1993; Ibarrola et al. 1998b). El ajuste de la capacidad digestiva ocurre a través de la modulación de i) la producción de lisosomas en las células digestivas, que lleva asociadas alteraciones de la altura del epitelio digestivo y el volumen total de la glándula digestiva (Ibarrola et al. 2000a) y ii) la actividad del pool enzimático del estilo cristalino y la glándula digestiva (Ibarrola et al. 1998b).

Actividad de los enzimas digestivos. Desde la década de los 60 del siglo pasado se ha publicado un considerable número de estudios relativos a la actividad de carbohidrasas, proteasas y lipasas en el tracto digestivo de los bivalvos (Purchon, 1978; Vonk and Western 1984; Ibarrola et al. 1998, 2012; Sakamoto and Toyohara 2009; Albentosa et al. 2012). En el aspecto funcional cabe destacar que el sistema digestivo de los bivalvos presenta una gran capacidad para modular tanto la actividad enzimática (cambios cuantitativos) como la composición del pool enzimático (cambios cualitativos) para ajustar el potencial digestivo a las variaciones de las características de la dieta. En trabajos realizados en nuestro laboratorio (Ibarrola et al. 1996, 1998, 1999, 2000a, b; Navarro et al. 2009; Arambalza et al. 2018) se ha demostrado que el berberecho *Cerastoderma edule* responde de manera muy rápida (tres días) a las alteraciones del contenido orgánico de la dieta ajustando el nivel total del conjunto de actividades enzimáticas y muy específicamente la actividad de la enzima celulasa. Respuestas similares también se han encontrado oen otras especies de bivalvos (Bayne, 1993; Fernández-Reiriz, 2001; Labarta et al. 2002).

Tiempo de paso del alimento por el tracto digestivo (*gut passage time*). Este parámetro hace referencia al tiempo durante el cual el material ingerido es retenido en el sistema digestivo y representa el tiempo efectivo para la digestión, hidrólisis y absorción del alimento. Experimentalmente, el tiempo de paso se determina incorporando carbono marcado radioactivamente al alimento. Siguiendo postulados teóricos acerca del funcionamiento del sistema digestivo de los animales (Taghon et al. 1978; Sibly y Calow, 1986), el tiempo de paso puede calcularse como el resultado de la división entre la capacidad digestiva (mg) y la tasa de ingestión (mg/h). Puesto que el tiempo de paso representa el tiempo del que dispone el digestivo para hidrolizar y absorber el alimento, éste parámetro determina la eficiencia bruta de absorción del alimento. La relación positiva entre tiempo de paso y eficiencia de absorción es una característica fundamental en los animales de alimentación continua como los bivalvos y ha sido corroborada experimentalmente con técnicas de marcaje radioactivo (Bayne et al. 1987, 1989; Navarro e Iglesias, 1993; Navarro et al. 1994; 2009; 2016). La digestión extracelular estomacal y la posterior selección digestiva influyen decisivamente en el tiempo de paso del alimento: una digestión extracelular intensa y/o una selección eficiente de partículas implicaría una mayor asignación de partículas a la glándula digestiva para su digestión intracelular y, consiguiente, una retención prolongada de la ingesta. Por el contrario, una digestión extracelular insuficiente y/o una baja eficiencia de selección digestiva resultaría en una menor incorporación de partículas a la glándula digestiva y la derivación de una mayor proporción de la ingesta al intestino para su egestión rápida (Ward & Shumway 2004). El tiempo de paso en los bivalvos es a su vez dependiente de la tasa de ingestión: en comedores continuos el incremento de la ingestión provoca la aceleración del tránsito intestinal dando lugar a la reducción del tiempo de paso del alimento y, por consiguiente, de la eficiencia de absorción (Winter 1978; Widdows et al. 1979; Møhlenberg and Kiørboe, 1981; Navarro and Winter, 1982; Bayne et al.1987; Bayne et al. 1989; Beiras et al. 1993; Navarro et al. 2009).

La eficiencia neta de absorción depende de todos los parámetros digestivos descritos anteriormente (capacidad digestiva, actividad enzimática, tiempo de retención). Dado el grado de interrelación de estos parámetros digestivos entre sí, así como sus múltiples dependencias con respecto a las características de la dieta, no es de extrañar que la eficiencia de absorción presente una enorme variabilidad en bivalvos

sometidos a condiciones alimenticias cambiantes. No obstante, se han podido establecer una serie de relaciones fundamentales.

i) La concentración del alimento en el medio esta inversamente relacionada con la eficiencia de absorción (Bayne 1973; Widdows 1978; Thompson & Bayne 1974). Está relación negativa esta principalmente condicionada por el efecto positivo ya descrito que la concentración de alimento ejerce sobre la tasa de ingestión. El incremento en la tasa de ingestión conlleva una reducción del tiempo de paso, lo que da lugar a la reducción de la eficiencia de absorción.

ii) La calidad del alimento ejerce un efecto positivo sobre la eficiencia de absorción. Tanto en el mejillón *Mytilus galloprovincialis* (Navarro et al. 1991) como en el berberecho *Cerastoderma edule* (Navarro et al. 1994; Urrutia et al. 1996, 2001) se ha observado que la eficiencia de absorción aumenta con el contenido orgánico de la ingesta siguiendo una tendencia que se ajusta a modelos curvilineales asintóticos. Esta dependencia se basa en el comportamiento de la capacidad digestiva que tiende a ser más alta cuanto mayor es el contenido orgánico del material ingerido (Navarro & Iglesias 1993; Ibarrola et al. 1998), debido probablemente al incremento de la fracción de ingesta que es seleccionada para ingresar en la glándula digestiva.

El efecto de la temperatura sobre la eficiencia de absorción sigue siendo objeto de controversia. A priori, el efecto directo de la temperatura sobre las reacciones químicas debería dar lugar a la existencia de una relación positiva entre eficiencia de absorción y temperatura. Sin embargo, tal y como se ha analizado previamente, la eficiencia de absorción es altamente dependiente de otros parámetros fisiológicos que, a su vez, también son dependientes de la temperatura. Por ejemplo, el potencial efecto positivo de la temperatura sobre la actividad digestiva podría verse compensado por la serie de efectos impuestos sobre la tasa de aclaramiento y, en consecuencia, sobre el tiempo de paso del alimento. Como se ha indicado anteriormente, la tasa de aclaramiento muestra una dependencia térmica muy acusada y, por tanto, el incremento de la temperatura puede dar lugar al incremento de la tasa de ingestión, lo que provocaría la reducción del tiempo de paso del alimento. Estas interrelaciones múltiples dificultan enormemente el análisis aislado del efecto térmico sobre la eficiencia de absorción, y por ello en la literatura pueden encontrase resultados contradictorios entre si, ya que se han descrito efectos térmicos tanto positivos, (Winter 1978), como

negativos (Widdows & Bayne 1971) o neutros (Wilbur & Hilbish, 1989) de la temperatura sobre la eficiencia de absorción.

2.2 Procesos de disipación de energía

2.2.1 Excreción

La pérdida de energía que se deriva de la excreción de restos nitrogenados (amonio) en la orina suponen, generalmente, una proporción minoritaria de la pérdida total de energía en los bivalvos. Se ha estimado que supone en torno a un 1-10% (dependiendo de la composición de la dieta entre otros factores que influyen a la excreción) del gasto metabólico total. Dado que su influencia sobre el balance energético es menor, es un parámetro fisiológico que habitualmente no se considera en las determinaciones del balance energético (Bayne and Newell 1983; Bayne et al. 1987; Beiras et al. 1995).

2.2.2. Metabolismo

Se entiende por metabolismo al conjunto de reacciones químicas que tienen lugar en el organismo. El metabolismo puede dividirse en a) la oxidación de los compuestos orgánicos mediante la utilización de oxígeno con la consiguiente liberación de energía libre de Gibbs y su utilización para sintetizar (refosforilar) ATP y b) la subsiguiente utilización del ATP para realizar el conjunto de trabajos o funciones vitales del organismo (trabajo mecánico, trabajo electroquímico y trabajo sintético). La energía invertida en la realización del conjunto de funciones vitales o trabajo metabólico se desprende del organismo en forma de calor. Así la determinación de la tasa metabólica requiere del cómputo de la cantidad de calor liberado por el organismo por unidad de tiempo. Sin embargo, determinar el calor producido por un organismo plantea obvias dificultades, y por lo tanto, dado que el oxígeno es el último aceptor de la cadena de electrones, en organismos aerobios la tasa metabólica se estima habitualmente a partir del consumo de oxígeno. A lo largo de este trabajo al referirnos a la tasa metabólica del organismo distinguiremos dos niveles diferentes: tasa metabólica estándar (SMR) y tasa metabólica de rutina (RMR).

La tasa metabólica estándar hace referencia a los gastos propios del mantenimiento del organismo para la homeostasia celular e integridad funcional. La práctica habitual para cuantificar el metabolismo estándar en los bivalvos consiste en someter al organismo a un periodo de ayuno por tiempo suficiente, en torno a 6-7 días

(Tamayo et al. 2011), como para minimizar cualquier gasto energético derivado de procesos digestivos. Los procesos que contribuyen en mayor grado al gasto metabolismo estándar en los bivalvos son fundamentalmente dos: i) la renovación proteica (*turnover protéico)* que representa el continúo ciclo de síntesis y degradación de las proteínas celulares, ii) el transporte transmembrana de Na^+ y K^+ mediante la acción de la ATP-asa sódico-potásica en la membrana plasmática y iii) la fuga de H^+ a través de la membrana mitocondrial. Se ha estimado (Hawkins & Bayne 1985; Hawkins et al.1986, 1989) que la renovación proteica puede contribuir tanto como un 70% al gasto metabólico estándar en el mejillón *Mytilus edulis*. La composición lipídica de las membranas celulares también tiene gran influencia en los costes metabólicos basales. En la ostra *Crassostrea gigas* se ha descrito una correlación significativa entre el metabolismo estándar y el grado de insaturación de fosfolípidos de las membranas (Pernet et al. 2006, 2007, 2008). La existencia de esta correlación sugiere que el transporte iónico transmembrana contribuye significativamente al metabolismo estándar.

El término metabolismo de rutina se utiliza para definir la actividad metabólica de un organismo en condiciones normales de actividad y alimentación. Por lo tanto, el metabolismo de rutina es el resultado de añadir, a los costes metabólicos estándar explicados anteriormente, los costes propios de adquisición y procesamiento del alimento y los asociados al incremento o síntesis de tejido somático o reproductivo (Parry, 1983). En los bivalvos los costes de estos procesos son comúnmente denominados costes de crecimiento, y dada la estrategia alimenticia de estos organismos (la ingestión continua), comprenden un incremento constante respecto al metabolismo estándar en ambientes donde el organismo puede alimentarse.

Al igual que los procesos de adquisición y absorción, los costes metabólicos (tanto estándar como de rutina) están influenciados por las condiciones ambientales del entorno donde el organismo desarrolla su actividad. Como en el apartado anterior, vamos a analizar el efecto que tanto el entorno nutricional como la temperatura ejercen sobre la tasa metabólica de los bivalvos.

Efecto que las condiciones nutricionales sobre la tasa metabólica. La existencia de una correlación positiva entre tasa de absorción y tasa metabólica en los bivalvos se ha puesto de manifiesto en numerosas ocasiones (Thompson and Bayne 1974; Bayne et al. 1989; Jorgensen 1990; Bayne 1999, 2000; Tamayo et al. 2014). Dada la relación

positiva entre concentración y contenido orgánico del alimento y la tasa de absorción descrita en el apartado anterior, se ha observado la existencia de una relación positiva entre concentración de materia orgánica en el medio y gasto metabólico (Griffiths and King 1979; Thomson and Bayne 1974; Hawkins et al. 1986; Albentosa et al. 2012). Los bivalvos que habitan las zonas intermareales del litoral están expuestos a períodos más o menos prolongados de exposición aérea, durante los cuales se encuentran privados de alimento y oxígeno. La exposición aérea es un factor limitante del crecimiento en las poblaciones intermareales de bivalvos (Peterson and Black 1988), de hecho, la tasa de crecimiento está inversamente relacionada con el tiempo de exposición al aire (Kopp, 1979). Por un lado, la exposición aérea interrumpe, obviamente, la ingestión de alimento, y si bien es cierto que la exposición aérea contribuye a retener durante más tiempo el alimento contenido en el tracto digestivo, el posible efecto beneficioso que de ello se derivaría sobre la eficiencia de absorción, no compensa el déficit de absorción provocado por la inanición durante la exposición al aire. Al suprimirse la actividad alimentaria, se produce una disminución considerable de la demanda de energía en los periodos de emersión que varía entre el 4 y 78% con respecto a la demanda en condiciones de inmersión (Bayne and Newell 1983; Griffiths and Griffiths 1987). Por otra parte, el déficit de consumo de oxígeno tiene importantes implicaciones en el metabolismo: se produce acumulación de CO_2, acidificación de los tejidos (Allen and Burnet 2008), transición al metabolismo anaerobio para responder a la demanda energética del organismo, así como desarrollo de hipometabolismo. Se han diferenciado dos claras estrategias de compensación fisiológica a la reducción en la disponibilidad de oxígeno durante la exposición aérea. Algunos bivalvos (por ejemplo, *Cerastoderma edule*) poseen la capacidad de adquirir oxigeno del aire mediante la apertura de valvas (denominada en inglés *air-gaping*), que les permite mantener el metabolismo en períodos de emersión a niveles altos con respecto a la tasa acuática y evitar el uso de ruta anaerobias. Otros bivalvos (por ejemplo, *Mytilus galloprovincialis*) no pueden obtener oxígeno del aire y recurren a una acusada supresión de la tasa metabólica como vía de compensación y minimización de acumulación de productos anaeróbicos (Widdows and Shick 1985).

Efecto de la temperatura sobre el metabolismo. En los organismos termo-concordantes, la tasa metabólica exhibe una dependencia térmica muy acusada. La temperatura altera la velocidad de los procesos físico-químicos tales como la velocidad

de las reacciones químicas, las tasas de difusión de moléculas, la fluidez de las membranas y la estructura de las proteínas (Hochaka and Somero 2002). Los trabajos de Portner y colaboradores (Portner, 1998, 2000, 2001) han demostrado que los límites de la tolerancia térmica (tanto inferior como superior) sobrevienen cuando el alcance aerobio para el metabolismo se reduce al mínimo como consecuencia del desequilibrio entre captación y demanda tisular de O_2 y, por consiguiente, los requerimientos metabólicos de los tejidos han de mantenerse mediante rutas anaeróbicas. En el límite térmico inferior el déficit de captación de oxígeno ocurre como consecuencia de la ralentización y finalmente el colapso de la función respiratoria, mientras que, en el límite térmico superior, se debe a la incapacidad del sistema circulatorio de satisfacer la exagerada demanda de oxígeno provocada por la aceleración del conjunto de procesos metabólicos. El rango de tolerancia térmica (*ventana térmica*) varía entre especies, y se ha asociado con la distribución latitudinal de las mismas (para una revisión más exhaustiva de estos conceptos ver Portner 2001, 2002 y 2010)

En el rango de tolerancia térmica existe una relación positiva entre temperatura y tasa metabólica que ha sido descrita en numerosos trabajos en diferentes especies de bivalvos (Shumway and Koehn 1982; Bayne and Newell 1983; Mac Donald and Thompson 1985; Resgalla et al. 2007; Kang et al. 2016). El aumento en el consumo de oxígeno tiene su origen en un aumento en la demanda energética del organismo, principalmente debido al aumento del metabolismo protéico, al aumento en los costes de ventilación y circulación y al aumento en la actividad de la bomba Na^+/K^+ ATPasa en respuesta al incremento en la permeabilidad del epitelio y membranas celulares (Moseley et al. 1994; Portner et al. 1999; Portner 2000, 2001). Cuando una exposición a un cambio en la temperatura se prolonga en el tiempo, se ha observado que los bivalvos poseen mecanismos de compensación (respuestas crónicas) que les permite optimizar sus procesos metabólicos paliando así el efecto de la temperatura y modificando los límites de tolerancia térmica. Estos mecanismos compensatorios afectan principalmente al metabolismo estándar y a los costes de mantenimiento (Portner 2010). Entre las modificaciones o adaptaciones llevadas a cabo por los organismos para minimizar el efecto de la temperatura destacan la modificación en la fluidez de las membranas mediante ajustes en el índice de insaturación de los fosfolípidos que las componen (Hulvert and Else 1999; 2004, Hall et al. 2002), ajustes en las propiedades cinéticas de las enzimas metabólicas y ajustes en los transportadores transmembrana.

3- Diferencias interindividuales en la tasa de crecimiento de los bivalvos

El comportamiento que muestran los moluscos bivalvos frente a las modificaciones que se producen en las condiciones ambientales puede calificarse de complejo, si atendemos al alto grado de plasticidad observado en las especies estudiadas. Son muchos los estudios que se han realizado en distintos ámbitos a lo largo de los años con el fin de analizar la variabilidad de esas respuestas y los factores que las determinan. Por un lado la caracterización de la variabilidad interespecífica ha sido objeto de estudio en numerosos trabajos centrados en analizar el efecto de distintas variables ambientales sobre el balance energético y sus principales componentes; así, por ejemplo, Widdows and Shick (1985) caracterizaron el efecto de la exposición aérea sobre el balance energético y sus componentes principales en las especies *Cerastoderma edule* y *Mytilus edulis;* Defosez and Hawkins (1997) analizaron y compararon la selección preingestiva en individuos de las especies *Mytilus edulis*, *Ruditapes philippinarum* y *Ruditapes decussatus*; y Velasco and Navarro (2002) cuantificaron los parámetros fisiológicos que gobiernan la entrada de energía en el organismo en las especies *Mulinia edulis* y *Mytilus chilensis* en función de la concentración y de la calidad del alimento en suspensión. La variabilidad interespecífica en la respuesta inmunológica también ha recibido especial interés, por ejemplo, Wooton et al. (2003) extrajeron hemolinfa del músculo de las especies *C.edule, M.edulis y Ensis siliqua* e hicieron una comparativa interespecífica de varios ensayos inmunológicos; Dang et al. (2013) analizaron el efecto de la infección por *Perkinsus* sobre el crecimiento y el índice de condición de las almejas *Ruditapes decussatus* y *Ruditapes philippinarum,* demostrando una correlación inversamente proporcional en ambas especies.

Esta alta variabilidad también se ha observado entre individuos de una misma especie. El análisis de los factores que determinan la alta variabilidad intraespecífica ha sido y sigue siendo objeto de estudio. Partiendo de la base de que el componente endógeno o genotípico tiene una gran influencia en las diferencias interindividuales en los moluscos bivalvos, la literatura referente a estas diferencias puede agruparse en dos bloques principales. Por un lado, la referente a estudios inter-poblacionales en los que determinadas características de individuos de distintas poblaciones de una misma especie son sometidas a estudio, como por ejemplo el crecimiento (Camacho et al. 1995, Navarro et al.1991, Ibarrola et al. 2017) o el grado de acumulación diferencial de contaminantes como indicador del estado del ecosistema donde habitan (Byrne &

O´halloran 2001; Hamza-Chaffai et al. 2003; Roméo et al.2003; Andral et al. 2004; Usero et al. 2005). Por otro lado, destaca la literatura que recoge la variabilidad existente entre individuos de una misma población, en condiciones en las que el aporte de los factores exógenos sobre dicha variabilidad es a priori mínimo, puesto que todos los individuos conviven bajo las mismas condiciones ambientales. En toxicología, se ha descrito en diferentes especies la existencia de diferencias interindividuales en la acumulación de metales pesados en correlación con la tasa de aclaramiento, índice de condición e incluso con el tamaño del organismo (Wang 2001; Mubiana et al. 2006). También se han observados respuestas diferenciales entre individuos sometidos a condiciones de estrés producidas por el descenso en la salinidad del agua y aumento de temperatura, condiciones que además magnifican las diferencias interindividuales que se observan en condiciones no estresantes (Lagus and Sukhotin 1998; Sukhotin et al. 2003).

Centrándonos en el crecimiento, que es el tema de estudio de esta tesis doctoral, está aceptado, desde la publicación del estudio de Singh y Zouros (1978) y trabajos posteriores (Zouros et al. 1980; Hawkins et al. 1986; Hawkins 1995; Bayne and Hawkins 1997), que el crecimiento diferencial observado entre individuos de una misma población está estrechamente correlacionado con el grado de heterocigosidad genética. El conocimiento de que la base genética subyace en las diferencias de tamaño entre individuos ha sido el punto de partida de muchos estudios que han tratado de establecer las bases genéticas de las diferencias inter-individuales en tasa de crecimiento, al objeto de mejorar la producción de la industria acuícola mediante la reducción del tiempo de cultivo necesario para alcanzar la talla deseada (Newkirk 1980; Crenshaw et al. 1991; Li et al. 2011). Los programas de selección de líneas de crecimiento han resultado eficaces en vieiras (Ibarra et al.1999; Zheng et al.2004), almejas (Hadley et al. 1991), mejillones (Toro et al. 2004, Alcapan et al. 2007) y ostras (Toro and Newkirk 1991; Li et al. 2011), y en las especies *Ostrea edulis* y *Crassostrea gigas* se ha estimado y/o observado un aumento en el crecimiento de en torno al 10% por generación (Newkirk 1980, Li et al. 2011).

En el presente trabajo hemos caracterizado los parámetros fisiológicos y las diferencias moleculares de individuos de alta y baja tasa de crecimiento de la especie *Mytilus galloprovincialis,* por lo que consideramos pertinente una revisión de la literatura

existente en relación a estos aspectos para contextualizar adecuadamente el estudio y facilitar la compresión de las diversas cuestiones que se discutirán en cada capítulo.

3.1. Bases fisiológicas de las diferencias interindividuales en la tasa de crecimiento

Los estudios de fisiología energética comparada entre individuos de alta y baja tasa de crecimiento de una misma población adquirieron cierta relevancia tras el descubrimiento del efecto significativo que ejerce el grado de heterocigosidad genética sobre el crecimiento (Singh and Zouros 1978) en la ostra *Crassostrea virginica*. Una parte importante de la alta variabilidad que se observa en moluscos bivalvos en las tasas de crecimiento de individuos de una misma población se puede deber, por lo tanto, a diferencias de carácter endógeno. La literatura disponible en este ámbito es abundante, así como lo es la diversidad de diseños experimentales y especies estudiadas. He considerado oportuno realizar aquí una breve descripción de los artículos más relevantes y más frecuentemente citados en este campo:

Garton et al. (1984) recolectaron semillas de almeja (*Mulinia lateralis)* de aproximadamente 6 mm en una población natural de la costa este de Estados Unidos; obtuvieron datos de crecimiento individual durante 4 semanas en el laboratorio e hicieron determinaciones del balance energético. Adicionalmente, cuantificaron el grado de heterocigosidad de cada individuo mediante electroforesis de 6 enzimas diferentes. Observaron una relación significativa entre heterocigosidad genética y crecimiento. Además, concluyeron que la mayor parte de las diferencias interindividuales en el crecimiento derivaba de las diferencias en el componente metabólico del balance de energía.

Hawkins y colaboradores (Hawkins et al. 1987; Hawkins and Day 1996) analizaron el balance energético de mejillones adultos (≈ 6-7 cm de longitud de concha) de la especie *Mytilus edulis* recolectados de una población natural. Estimaron el balance energético (SFG) a nivel individual y cuantificaron diversos componentes del metabolismo del nitrógeno tales como la tasa de síntesis de proteínas o la actividad de enzimas proteolíticos. Concluyeron que los individuos con mayores SFG eran aquellos que presentaban costes metabólicos más reducidos, y observaron que el menor gasto metabólico se asociaba a un metabolismo proteico más eficiente.

Toro et al. (1996) produjeron varias cohortes de la ostra *Ostrea chilensis* a partir de parentales recolectados en el medio natural. Analizaron la correlación de los distintos parámetros fisiológicos de los individuos con el grado de heterocigosidad tanto en juveniles de 10 meses de edad (0.1 y 1.5 gramos de peso vivo) como en adultos de 36 meses de edad (8.75 y 47 gramos de peso vivo) y mostraron que, en juveniles, los parámetros fisiológicos que gobiernan la adquisición de energía (tasa de ingestión orgánica (OIR), tasa de absorción (AR) e incluso el Scope for Growth (SFG)) se correlacionaban significativamente con el grado de heterocigosidad, mientras que, por el contrario, en los adultos era la tasa metabólica el único parámetro fisiológico correlacionado significativamente con el grado de heterocigosidad.

Bayne et al. (1999a) realizaron tres experimentos con progenies obtenidas de cruces entre parentales de distintas familias de la ostra *Crassostres gigas*, en las que midieron los parámetros fisiológicos del balance energético en ejemplares sometidos a distintas condiciones ambientales. Observaron que la mayoría de los individuos provenientes de cruce entre familias (híbridos) presentaban mayores tasas de adquisición y similares costes metabólicos que las familias progenitoras. Además, compararon también los parámetros fisiológicos entre los individuos cuyos tamaños se situaban en la cabeza (F: fast growers) y la cola (S: slow growers) de la distribución de tamaño de una de las poblaciones, siendo la diferencia en peso entre ambos grupos de aproximadamente 3x (F: ≈0.25 g; S.≈ 0.09 g de peso seco). La tasa de aclaramiento (estandarizada a un peso común de 0.19g de peso seco) de los individuos de alta tasa de crecimiento duplicaba la de los individuos de baja tasa de crecimiento (F: 0.55 l/h; S: 0.28 l/h). Bayne et al. (1999b), analizaron las bases fisiológicas del crecimiento diferencial en la ostra *Saccrostrea commercialis*. Para ello compararon el balance energético de individuos provenientes de una población natural con el de individuos obtenidos de un programa de selección para la mejora de la especie. La serie de experimentos incluía la determinación de los parámetros del balance en individuos sometidos a distintas concentraciones de alimento. Los individuos seleccionados mostraron mayores tasas de aclaramiento, y menores costes metabólicos de alimentación y crecimiento que los individuos no seleccionados (0.24 vs 0.48 Joules gastados por Joule ingerido en los individuos seleccionados y no seleccionados respectivamente).

Pace et al. (2006) realizaron cruces con ejemplares de la ostra de especie *Crassostrea gigas* mediante los que obtuvieron una serie de familias (35) de larvas que cubrían un amplio espectro de diversidad genética y presentaban una amplia variabilidad en tasa de crecimiento. El análisis de las variables fisiológicas del balance energético mostró que las diferencias en tasa de crecimiento entre las familias de larvas estaban asociadas a diferencias en i) la capacidad para adquirir alimento (filtración) y ii) los gastos metabólicos del crecimiento que se derivarían, probablemente, de diferencias en el coste metabólico de la deposición de proteínas.

Tamayo et al. (2011) analizaron las bases fisiológicas del crecimiento diferencial en individuos de la almeja *Ruditapes philippinarum* producidos en hatchery. Se realizaron dos aproximaciones diferentes para abordar el estudio de los parámetros fisiológicos del balance energético en semillas de almeja que presentan diferencias en tamaño. Por un lado, se utilizaron individuos de alta (F) y baja (S) tasa de crecimiento provenientes de una misma cohorte, con una diferencia en tamaño de aproximadamente 3x (F: 0.29 g y S 0.09 g de peso vivo) y por otro, se utilizaron individuos de tamaño similar perteneciente a dos cohortes distintas (edad: 170 y 390 días). En ambos experimentos, los individuos de crecimiento rápido poseían, con respecto a los de crecimiento lento, mayores tasas de aclaramiento y menores costes metabólicos de alimentación y crecimiento. En un trabajo posterior (Tamayo et al. 2014) obtuvieron individuos de una misma cohorte de *Crassostrea gigas* que presentaban unas diferencias muy elevadas en tamaño. Los individuos de alta tasa de crecimiento tenían un peso vivo de ≈1.9 gramos y una longitud de concha de ≈24 mm, mientras que el peso y la longitud de los individuos S era de 0.05 gramos y 6 mm. Las determinaciones de los componentes del balance energético confirmaron los hallazgos anteriores, los individuos F poseían una mayor capacidad filtradora, así como una mayor eficiencia metabólica. Además, basándose en el diferente exponente alométrico que determina la dependencia de la tasa metabólica y la tasa de aclaramiento en los bivalvos, y modelizando ambos parámetros fisiológicos para un amplio rango de tamaños, estos autores sugirieron que la talla asintótica máxima, en la que el balance energético alcanzaría el valor de 0, sería superior en los individuos F que en los individuos S. En otras palabras, los individuos S perderían la capacidad para crecer a tamaños inferiores que los individuos F.

Por ultimo, Ibarrola et al. (2017) realizaron un estudio en el que compararon los parámetros fisiológicos de 6 familias del mejillón *Perna canaliculus.* La comparativa

inter e intra-familiar desveló que los individuos de crecimiento rápido combinaban una capacidad filtradora mayor con una mayor eficiencia metabólica, en concordancia con resultados precedentes. Estos autores observaron además que el peso de la glándula digestiva, estandarizada a un peso común, de los individuos F era menor que la de los S. Este hallazgo sugeriría en primera instancia que los individuos de crecimiento rápido presentarían una capacidad para albergar y retener alimento inferior a la correspondiente a los individuos de crecimiento lento. Sin embargo, tal y como indican los autores, se pudo comprobar que si bien ciertamente con carácter general los animales con mayores glándulas digestivas presentaban tasas de aclaramiento y por lo tanto de procesamiento del alimento más elevadas, los individuos identificados como F se caracterizaban por un procesamiento digestivo más eficiente que los individuos S, puesto que podían procesar en glándulas digestivas de menor tamaño una mayor cantidad de alimento filtrado por unidad de tiempo sin merma en la eficiencia de los procesos digestivos.

Prácticamente todos los estudios de fisiología energética muestran que el crecimiento diferencial entre individuos de crecimiento rápido (F) y lento (S) se basa en la existencia de diferencias endógenas o innatas bien en la capacidad para adquirir y/o procesar alimento, bien en el gasto metabólico asociado a los procesos basales o los procesos de adquisición, procesamiento y absorción del alimento y la deposición de nuevo tejido. Bayne et al. (1999b) compendió la información existente en este campo y propuso tres modelos explicativos, no mutuamente excluyentes, de las causas fisiológicas que dan lugar a la variabilidad interindividual en el crecimiento. Estos tres modelos de Bayne constituyen un marco intelectual para la interpretación de la información, a veces contradictoria, que proviene de los diversos estudios que utilizan la aproximación energética para analizar las bases biológicas de las diferencias inter-individuales en tasa de crecimiento. Aunque estos modelos se definieron hace ahora casi 20 años, tienen hoy día plena vigencia. Por ello, hemos considerado oportuno presentar aquí los tres modelos propuestos por Bayne (1999b) y describir los trabajos publicados que se ajustarían a cada uno de los mismos.

Modelo de adquisición de energía (*acquisition model)*

El modelo de adquisición establece las diferencias interindividuales en la capacidad para crecer se derivan de la existencia de diferencias endógenas (genéticamente establecidas) en la capacidad o eficiencia de los mecanismos

fisiológicos implicados en la adquisición de energía. Es decir, básicamente, se entiende que las diferencias en la capacidad para crecer se derivan de la existencia de diferencias interindividuales en la capacidad para, i) bombear agua, ii) filtrar y retener partículas en las branquias, iii) seleccionar las partículas orgánicas en los palpos labiales iv) albergar e hidrolizar las partículas y v) absorber los monómeros resultantes de la digestión.

Son varios los estudios que han descrito que los individuos de alta tasa de crecimiento poseen una mayor capacidad filtradora, y por consiguiente de ingesta, sin provocar una disminución en la eficiencia de absorción. Toro et al. (1996) y Toro and Vergara (1998) hallaron evidencias de esta naturaleza en juveniles de la ostra *Ostrea chilensis*. Mayores tasas de adquisición de alimento también han sido descritas en la ostra *Saccrostrea gigas* (Bayne et al.1999 b) y en diferentes estadios de la ostra *Crassostrea gigas* (Bayne et al. 1999a; Bayne 1999; Pace et al. 2006; Tamayo et al. 2014). Tanto en juveniles de mejillones *Mytilus galloprovincialis* como en almejas *Ruditapes philippinarum* los individuos de alta tasa de crecimiento también se caracterizan por combinar mayores tasas de ingestión, con la capacidad de mantener la eficiencia de absorción a niveles equivalentes a los de los animales de baja tasa de crecimiento, que muestran tasas de ingestión menores (Tamayo et al. 2011, 2013, 2015, 2016).

Los trabajos anteriormente citados ponen de manifiesto la importancia de la capacidad endógena de desarrollar mayores tasas de filtración sin merma de la eficiencia de los procesos de absorción; sin embargo, las causas que promueven tales diferencias endógenas no están claras. Tamayo et al. (2011) encontraron una estrecha relación entre tasa de aclaramiento y el área de la superficie branquial: los individuos que desarrollaban tasas de filtración más elevadas poseían también branquias significativamente mayores. El trabajo de Tamayo et al (2011) plantea, por lo tanto, que la diferencia en capacidad filtradora podría estar asociada a diferencias de carácter anatómico: el tamaño de las branquias. No obstante, puesto que como ya se ha descrito con anterioridad, existe un compromiso funcional entre eficiencia de absorción y tasa de ingestión, la capacidad para adquirir más alimento no tendría necesariamente que redundar en mayores tasas de absorción de no existir un incremento en la capacidad digestiva que acompañe a las mayores superficies branquiales. No hay evidencias de que tal cosa suceda, pero en la ostra *Crassostrea gigas,* por ejemplo, si se ha observado que la actividad enzimática en la glándula digestiva, en concreto la actividad digestiva

más intensa, la amilasa, se correlaciona de manera positiva con la tasa de crecimiento (Prudence et al. 2006; Huvet et al. 2008).

En cuanto a la capacidad para seleccionar el alimento, pesar de que en los ecosistemas en los que habitan los bivalvos es bastante habitual que bajo determinadas condiciones estos animales recurran a la formación de pseudoheces, y pese a que se reconoce que la producción de pseudoheces constituye una estrategia de gran valor adaptativo tal y como hemos explicado anteriormente, el estudio de las potenciales diferencias que en este proceso pudieran existir entre individuos de alta y baja tasa de crecimiento ha recibido, hasta el momento, muy poca atención. En los únicos dos trabajos que abordan esta cuestión, publicados por Bayne et al. (1999a) y Bayne (2004) se encontró que los individuos de alta tasa de crecimiento de juveniles de ostra *Crassotrea gigas* y *Saccostrea glomerata* presentaban mayores eficiencias de selección preingestiva que aquellos de baja tasa de crecimiento.

Modelo de distribución de energía (*Allocation model)*

El modelo de distribución de energía se basa en la práctica, en el supuesto de que el gasto energético correspondiente a los procesos de mantenimiento de los individuos de alta tasa de crecimiento es comparativamente menor que en los individuos de baja tasa de crecimiento, de modo qué en términos de balance de energía, los primeros disponen de un mayor remanente energético que puede ser destinado al crecimiento, esto es, al incremento de tamaño. A día de hoy esta aceptado el coste de mantenimiento comparativamente menor de los organismos F se deriva de una mayor eficiencia en el *turnover protéico*, lo que supone asignar una menor cantidad de energía para esos procesos en comparación con los individuos S (Hawkins and Day 1996, 1999; Bayne and Hawkins 1997). Se han constatado diferencias significativas en los costes de mantenimiento en *Crassostrea gigas* (Bayne, 1999), *Crassostrea virginica* (Pernet et al. 2008), *Mytilus edulis* (Bayne and Hawkins 1997) y *Mytilus galloprovincialis* (Tamayo et al. 2016).

Modelo de eficiencia metabólica (*Metabolic efficiency model)*

El modelo de eficiencia metabólica sostiene que las diferencias en las tasas de crecimiento entre individuos F y S se basan en la diferencia del coste energético por

unidad de crecimiento, esto es, en la eficiencia de la inversión energética del proceso de síntesis de nuevos tejidos. Menores costes de crecimiento en los individuos de rápida tasa de crecimiento se han descrito en un gran número de trabajos (Toro and Vergara 1998; Garton et al. 1984; Bayne and Hawkins 1997; Bayne et al. 1999a, 1999b; Bayne 1999, 2000; Pace et al. 2006; Tamayo et al. 2011, 2013, 2014); además, en una parte importante de estos estudios, las diferencias en coste de crecimiento entre individuos suelen aparecer asociadas también a diferencias en la capacidad de adquisición de energía (Modelo I). Por ejemplo, Tamayo et al. (2014) basándose en la comparación de los datos de gasto metabólico de individuos de alta y baja tasa de crecimiento de juveniles de la especie *Crassostrea gigas* por unidad de tasa de absorción, concluyeron que los costes de crecimiento en los individuos S (0.59 Joules por Joule absorbido) eran 3 veces superiores a los costes de crecimiento en los individuos F (0.17 Joules por Joule absorbido).

A pesar de todo lo descrito, y de la cantidad de trabajos enfocados a la caracterización de las bases fisiológicas responsables en las diferencias interindividuales en la tasa de crecimiento de los bivalvos, no existe aun consenso sobre las causas fisiológicas de dicha variabilidad. Como se puede derivar de la revisión bibliográfica previamente presentada, la utilización de diferentes poblaciones, añadida a la heterogeneidad en los diseños experimentales en los trabajos, puede estar en el origen de las diferencias en las contribuciones relativas que los distintos autores han asignado a unos u otros procesos fisiológicos en la determinación de las diferencias intra-especificas en tasa de crecimiento. Recientemente, Tamayo et al (2016) sugirieron que los parámetros fisiológicos responsables de las diferencias entre individuos de alta y baja tasa de crecimiento estarían determinados por las condiciones ambientales en las se desarrollan los organismos. Estos autores recolectaron individuos de la especie *Mytilus galloprovincialis* de una población intermareal y, en el laboratorio, se dividieron en dos grupos de tamaño homogéneo. Cada grupo fue mantenido bajo distintas condiciones de alimentación, y se llevó a cabo un seguimiento del crecimiento individual para seleccionar, al cabo de un tiempo, aquellos individuos que presentaban las máximas diferencias en tasa de crecimiento (F, fast growers y S, slow growers). La diferencia entre las condiciones alimentarias de los dos grupos radicaba en la disponibilidad de alimento; un grupo fue alimentado de manera continuada con una dieta fitoplanctónica de alto contenido orgánico mientras que el otro grupo fue sometido a una fuerte

restricción alimentaria que consistía en que la misma dieta se le suministraba un día a la semana. Los experimentos de fisiología energética que se hicieron con los individuos F y S seleccionados en ambas condiciones de mantenimiento desvelaron que las bases fisiológicas que explicaban las diferencias en tasa de crecimiento entre individuos F y S eran distintas en las dos condiciones ambientales testadas. Los individuos de crecimiento rápido segregados en condiciones de alimentación continua presentaban mayores tasas de aclaramiento y menores costes de crecimiento que los individuos de crecimiento lento. Sin embargo, en el grupo de mejillones sometidos a alimentación discontinua, no se encontraron tales diferencias entre los individuos F y S, los individuos F, en ese caso, se caracterizaban por tener menores costes metabólicos estándar. Por lo tanto, parece evidente que al menos parcialmente, la disparidad de resultados reportados en la literatura referentes a las bases fisiológicas de la variabilidad interindividual en el crecimiento podría explicarse como consecuencia de la heterogeneidad de las condiciones en las que los organismos en estudio se desarrollaron como individuos de alta y baja tasa de crecimiento respectivamente.

3.2- Bases moleculares de las diferencias interindividuales en la tasa de crecimiento

A pesar del importante avance que se ha producido en las técnicas y herramientas de análisis molecular durante estas últimas décadas, aún existe un gran desconocimiento acerca de las bases moleculares de las diferencias interindividuales en tasa de crecimiento de los moluscos bivalvos. Los pocos estudios moleculares realizados en crecimiento de bivalvos esta en contraposición de la tendencia al alza de la producción acuícola de estos organismos y de la abundancia de estudios realizados en osteictios, la principal especie producida en la acuicultura. (Saavedra and Bachere 2006; Tanguy et al. 2008; Astorga et al. 2014). La escasa información genómica de la que se dispone, sin duda, es una de las principales razones del paulatino avance en este ámbito. A pesar de esta contradicción entre datos de productividad y conocimiento, la información genómica aumenta día a día, lo que presumiblemente facilitará diseños experimentales enfocados a descifrar la base molecular del crecimiento en bivalvos. De hecho, ya en 2012 se publicó el genoma de la especie *Crassostrea* gigas y recientemente, en 2017 se han publicado los genomas de la ostra *Crassostrea Virgínica* y de la vieira *Mizyhopecten yessoensis*. Aunque al parecer la publicación del genoma de

Mytilus galloprovincialis (la especie de estudio en el presente trabajo) está próxima, a día de hoy no ha acontecido.

Los primeros estudios en analizar las diferencias moleculares entre individuos de alta y baja tasa de crecimiento se centraron principalmente en analizar la heterosis genómica diferencial de los individuos (Singh and Zouros 1978; Zouros et al.1980; Hawkins et al. 1986; Hawkins 1995; Bayne and Hawkins 1997). Esta heterosis, fenómeno conocido como *vigor hibrido*, hace referencia a un mayor grado de heterocigosidad genética en los individuos que presentan mayores tasas de crecimiento como resultado de una mejor capacidad de adaptación y mayor grado de éxito (Mitton and Grant 1984). Dicho de otra manera, disponer de una mayor diversidad genética otorga a los organismos más heterocigotos una batería más amplia de genes que pueden expresar, y esto se traduce en un funcionamiento más eficiente y en mayores tasas de crecimiento que las de sus congéneres más homocigotos. En estos estudios se establecieron correlaciones significativas entre el grado de heterocigosidad y el crecimiento, e incluso se hallaron evidencias de una mayor eficiencia metabólica en los individuos más heterocigotos, asociada probablemente a menores costes del *turnover de proteinas*. Pernet et al. (2008) constataron que los individuos de alta tasa de crecimiento de la ostra *Crassostrea virginica* presentaban mayores grados de heterocigosidad. Además, estos autores pusieron de manifiesto una mayor eficiencia en la modificación del grado de insaturación de fosfolípidos de las membranas celulares frente a cambios de la temperatura ambiental en los individuos más heterocigotos (los de alta tasa de crecimiento), que implicaban menores costes metabólicos estándar con respecto a los organismos de menor tasa de crecimiento con mayor grado de homocigosidad.

La pérdida de cromosomas en las células también parece tener estrecha relación con la tasa de crecimiento en los bivalvos: Leitao et al. (2001) y Texeira da Sousa et al. (2011) describieron un mayor grado de aneuploidias en las células branquiales en los individuos de baja tasa de crecimiento en las especies *Crassostrea gigas* y *Ruditapes philippinarum,* y observaron una alta correlación negativa entre el crecimiento y el porcentaje de células afectadas por aneuploidias. Estos autores no obtuvieron información sobre los cromosomas que se perdían, pero en esa pérdida de información genética podría estar la clave que explicara la menor capacidad filtradora que habitualmente se observa en los organismos de baja tasa de crecimiento.

Todos estos trabajos evidencian una base molecular implícita en las diferencias fisiológicas que explican las diferencias interindividuales en los moluscos bivalvos. Como ya hemos adelantado anteriormente, el progreso en las técnicas de análisis molecular de la última década ha sido muy elevado, y por lo tanto las aproximaciones metodológicas para analizar las posibles diferencias moleculares han avanzado notablemente. Si bien es cierto que el estudio del genoma sigue siendo pertinente (e.g. búsqueda de SNP con carácter de marcador genético ó descripción de genoma completo), el análisis del transcriptoma ha adquirido gran relevancia en los estudios moleculares. A día de hoy son tres los métodos comúnmente utilizados para la comparación de la expresión génica o transcriptoma: 1- PCR en tiempo real ó qPCR, consistente en la obtención del nivel de expresión de genes concretos mediante el uso de la PCR. La qPCR es muy ventajosa en términos temporales y económicos ya que se obtiene información sobre los genes en estudio de manera relativamente rápida, y a un coste mucho menor que con los otros dos métodos. 2- *Next generation sequencing (NGS).* Este término engloba diferentes técnicas de secuenciación masiva, que consiste en la secuenciación de todo del ARN de las muestras en estudio. Existen métodos diferentes (Illumina, 454, Solid sequencing) de secuenciación masiva; si bien no se trata aquí de hacer una revisión exhaustiva sobre las ventajas y/o desventajas que presenta cada método, cabe destacar que el uso de *NGS* proporciona una caracterización completa de la transcripción de genes en el organismo en el momento de la obtención de la muestra. Sin embargo, el volumen de datos que se obtiene es inmenso (millones de secuencias) y su tratamiento requiere un amplio conocimiento de programación y bioinformatica. Además, su coste económico sigue siendo muy elevado. 3- *Microarrays* de expresión. El uso de *microarrays* permite el análisis de expresión de un gran número de secuencias. Brevemente, la técnica se basa en la inclusión de sondas específicas en una superficie solida a la que se unen las moléculas expresadas en la muestra experimental, generando una señal fluorescente. La intensidad de esa señal determina el numero de uniones, y por lo tanto el nivel de expresión. Al igual que en las técnicas *NGS,* las opciones disponibles a la hora de plantearse analizar la expresión de ADN mediante microarrays son muy amplias: Tipo de array, número de secuencias, número de canales, etc. La decisión de qué tipo de array usar está sujeto principalmente a los objetivos de cada estudio. La principal desventaja de esta técnica es la obtención de una versión parcial de transcriptoma con respecto a cualquier análisis NGS. Sin embargo, el análisis de los datos obtenidos no requiere un amplio conocimiento de programación, e

incluso existen protocolos para varios programas o paquetes estadísticos (e.g. el paquete Limma de Bioconductor). Por lo tanto, el uso de microarrays es la opción de compromiso óptima entre analizar la expresión de genes concretos, lo cual requiere un riguroso conocimiento sobre que se está buscando, y un análisis completo del transcriptoma (NGS). En el presente trabajo, hemos considerado oportuno hacer el análisis de expresión diferencial mediante microarrays para avanzar en la comprensión las bases moleculares de las diferencias interindividuales en el crecimiento (capítulo 4).

Para concluir esta introducción, resulta pertinente hacer una somera revisión de los resultados que han ampliado la información genómica relativa a procesos relacionados con el crecimiento durante los últimos años, haciendo especial hincapié en los pocos que se han centrado en caracterizar las bases moleculares de las diferencias entre individuos F y S. Zhang et al. (2012) analizaron el transcriptoma de la ostra *Crassotrea gigas* mediante secuenciación masiva; centraron gran parte de su análisis en caracterizar los procesos involucrados en la formación de la concha, y hallaron 259 proteínas implicadas en la construcción y modificación de la matriz extracelular, por ejemplo Colágeno, Laminina y Fibronectina. Recientemente, Bassim et al. (2014) monitorizaron, mediante un microarray diseñado a partir de sus propios datos de NGS, la expresión génica del mejillón *Mytilus edulis* durante varias etapas de su desarrollo e identificaron varios grupos de genes relacionados con procesos de crecimiento (e.g. GATAD1, PIP5K1A, y ATRX). Recientemente han propuesto 29 genes como marcadores del crecimiento en esta especie (Bassim et al.2015).

El estudio realizado por Meyer & Manahan (2010) fue de los primeros en analizar la expresión diferencial de un gran número de genes entre individuos de alta y de baja tasa de crecimiento, por lo que es uno de los referentes en este ámbito. Analizaron en nivel de expresión de 350 genes candidatos (Hedgecock et al. 2007) en 4 familias de la ostra *Crassotres gigas*. Hallaron diferencias moleculares en genes relacionados con i) actividad alimentaria (ScPB), ii) metabolismo energético (e.g. ND4L y ATP-synthase 8). Además, un alto porcentaje de los genes diferencialmente expresados correspondían a procesos relacionados con el iii) metabolismo proteico, reforzando la importancia del *turnover proteico* en la diferenciación en tamaño de individuos F y S. Valenzuela- Miranda et al (2015), analizaron mediante *NGS* diferencias en el transcriptoma de la especie *Haliotis rufescens,* y publicaron una lista de los genes sobre-expresados más relevantes en cada uno de los fenotipos, entre los

que destaca la sobreexpresión en los individuos F de los genes Dineína, Colágeno y Laminina. De la Peña et al (2016) monitorizaron mediante qPCR la expresión de dos ferritinas en individuos de alta y baja tasa de crecimiento de la especie *Argopecten purpuratus* en 5 estadios de vida. Sugirieron que la ferritina *Apfer I*, era un potencial marcador de crecimiento entre individuos F y S. Wilson et al (2016) describieron una sobre-expression de genes relacionados con el metabolismo (ATPasa y sintasa de ácidos grasos) y con el metabolismo proteico en los individuos F, en concordancia con anteriores estudios. Finalmente, Saavedra et al. (2017) analizaron la expresión génica diferencial en la branquia y la glándula digestiva en individuos F y S de la especie *Ruditapes decussatus,* y sugirieron que las diferencias en la tasa de crecimiento entre estos individuos podrían deberse a la expresión diferencial de genes relacionados con el metabolismo energético e incluso con el sistema inmune.

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Chapter 1

Is fast growing in mussels reared under turbid conditions based on the same physiological performance than in mussels reared at clear waters?

Abstract

Interindividual variability in the growth rate of bivalves reared under the same environmental conditions mainly relies on genetically determined differences in the physiological performance underlying the energy balance. However, as it has been recently reported, the phenotypic features that are responsible for the fast or slow growing may vary depending on the nurturing conditions.

The aim of this study was to test if conditioning of mussels to nutritional environments forcing mussels to develop completely different feeding strategies (one above the pseudofeces production threshold and the other one below it), could lead mussels selected at those reference conditions as fast and slow growers to differ in their physiological profiles. So, with the goal of ascertaining the point to which turning to pseudofeces production could determine potential differences between F and S mussels with respect to their F and S counterparts never facing high concentration of particles, 400 mussel seeds were collected from an intertidal area to be maintained at the laboratory under two very different nutritional conditions: 200 mussels were fed a high organic content diet dosed below the pseudofaeces threshold (BP maintenance condition) whereas the 200 left were fed a low organic content diet dosed above the pseudofaeces threshold (AP maintenance condition); and after 3 months of maintenance, fast and slow growing mussel groups were selected from each rearing condition and denoted as: F_{BP}, S_{BP}, F_{AP}, S_{AP}.

Individuals from those 4 groups were then conditioned during one week to different experimental diets and the physiological parameters that determined the energy budget were measured. We found that the rearing condition exerted a minor effect on

the physiological parameters determining growth rate. As a rule, F mussels (F_{BP} and F_{AP}) displayed higher clearance rates (and also higher pre-ingestive selection efficiencies under turbid conditions), which combined to similar absorption efficiencies resulted in higher rates of food absorption; since no significant differences were observed in metabolic rates, fast growers achieved higher Scope for Growth (SFG: J/h) values than S mussels (S_{BP} and S_{AP}). F individuals were found to have higher gill surface areas than S individuals, irrespective of the rearing conditions, supporting previous findings suggesting a major role of the gill in the inter-individual growth rate differences in mussels.

Keywords: Fast growing, Clearance Rate, Absorption Efficiency, Scope for Growth, gill surface area

Introduction

Large differences in the growth rate observed between individuals reared under identical environmental conditions have provided strong evidences about the existence of genetically determined inter-individual variation in the growth potential of bivalves (Garton et al. 1984; Toro et al. 1996; Toro and Vergara 1998; Bayne et al. 1999a, b; Pace et al. 2006; Pernet et al. 2008; Tamayo et al. 2011, 2013, 2014, 2015, 2016; Fernández-Reiriz et al. 2016; Ibarrola et al. 2017). Intra-populational growth heterogeneity relies on the existence of significant inter-individual differences in the energy balance caused by endogenously determined variations in the feeding rate, which are often accompanied by differences in the energy cost of tissue deposition and growth and/or the basal or standard metabolic requirements of individuals (Bayne et al. 1999a, b; Bayne 2004; Pernet et al. 2008; Tamayo et al. 2011, 2016).

Currently it is out of debate that inter-individual differences between fast and slow growers may have a genetic origin (Hedgecock et al. 1996; Ditman et al. 1998), but nurture conditions might also play a key role in determining the phenotypic features driving fast growing. Tamayo et al. (2016) selected fast and slow growing juvenile mussels that were reared under two different nutritional conditions: one group of mussels was reared in conditions of continuous provision of phytoplankton (optimal conditions), while the other group was reared under a certainly restrictive nutritional condition (mussels were fed only 12h/day a low ration of phytoplankton). They found

that the physiological basis of fast growing was different between both groups. In mussels grown under optimal feeding conditions, fast growers differed from slow growers in their higher feeding rates and lower costs of growth. However, in mussels reared under restrictive feeding conditions, the advantageous innate feature in fast growers was their capacity to have reduced standard metabolic rates; it was concluded that the nurture conditions under which inter-individual size-differentiation occurs alters the nature of the physiological components acting on size differentiation of fast- versus slow-growing individuals.

The existence of broad differences in the feeding rates between fast and slow growing individuals has been reported in so many occasions (Toro et al. 1996; Toro and Vergara 1998; Bayne et al. 1999a, b; Pace et al. 2006; Tamayo et al. 2011, 2013, 2014, 2015; Fernández-Reiriz et al. 2016) that it might be concluded that a higher capacity for suspension feeding is a prevalent distinctive feature of fast growing individuals. Suspension feeding of bivalves is a complex process that implies the pumping and filtration of the seawater through the gills to capture suspended particles and, under certain conditions of high particle concentration (above the pseudofaeces production threshold), also the preingestive sorting of particles that may operate with the pseudofaeces formation.

Eco-physiological studies have demonstrated that modulation of the selective activity at the gill and/or the labial palps is a key step in the physiological response of bivalves to the continuous changes in the quality (organic proportion) and quantity (particle concentration in the suspension) of food that typically occurs at littoral environments (Bayne et al. 1989; Cranford and Gordon, 1992; Iglesias et al. 1992, 1996; Navarro and Iglesias, 1993; Urrutia et al. 1996; Barillé et al. 1997; Hawkins et al. 1998; MacDonald et al. 1998; Ibarrola et al. 2000; Bayne, 2002). In specimens fed naturally occurring heterogeneous mixtures of particles, the preferential rejection of organically-poor particles within the pseudofaeces promotes the organic enrichment of ingested food (Kiørboe and Møhlenberg 1981; Newell and Jordan, 1983; Bricelj and Malouf, 1984; Iglesias et al. 1992; Ward and MacDonald 1996; see Ward and Shumway 2004 for review), contributing thus to increase the efficiency of the subsequent processes of digestion and absorption. Pseudofaeces production and the consubstantial selective ingestion is, thus, an essential component of the feeding physiology and the scope for growth in bivalves. However, and in spite of the mighty impact that selective

feeding has on the energy balance in bivalves, there is scarce information about the potential contribution that inter-individual differences in the capacity for preingestive processing of food could have in determining inter-individual growth rate differences in bivalves. There is only a couple of studies (Bayne et al. 1999a; Bayne 2004) involving the comparison of feeding parameters of fast versus slow growing oysters (*Crassotrea gigas* and *Saccostrea glomerata* respectively) in nutritional conditions above pseudofaeces threshold, where it was found that fast growing was based on higher preingestive selection efficiencies associated to their higher clearance rates.

This lack of data about the potential effect that eventual differences in such a key feature of the feeding behavior of mussels could exert on the physiological phenotype determining differential growing rates lead us to design the experiments reported in this study, where fast (F) and slow (S) growing mussels seeds were selected after being reared under two different *maintenance conditions*, one of them resembling a turbid environment composed mainly of inorganic particles, which forced mussels to continuously produce pseudofaeces (named AP: above pseudofaeces) and another one representative of clear waters, where particle concentration was low and particle quality high, at which pseudofaeces were never produced (named BP: below pseudofaeces). By rearing the mussels under so different feeding environments we aimed to evaluate endogenously determine putative differences in the capacity for preingestive sorting and pseudofaeces production as a potential factor contributing to inter-individual differences in growth potential. We hypothesized that,

i) A better (endogenous based) performance at the preingestive level would be one of the key features of the physiological profile of mussels arising as fast growers at the AP condition (F_{AP}) by comparison with mussels found to be slow growers (S_{AP}).

ii) The physiological basis of fast growing would be different between mussels reared at nutritional conditions above or below the pseudofaeces production threshold

iii) Differential physiological traits driven by different nurturing conditions would be persistent enough so as to make mussels reared at both maintenance conditions to respond differentially when exposed to changes in the quality of quantity of the food.

Material and Methods

Experimental design

Aproximately 400 juveniles of 10 mm shell length of the mussel *Mytilus galloprovincialis* were collected from the rocky shore in Antzoras (Bizcay, North Spain, 43°24'29.1"N; 2°40'51.0"W) in February 2014. At the laboratory, mussels were separated into two groups of 200 individuals. Both groups were maintained in two sea water containing tanks at constant temperature (16 ºC) and water salinity (33 PSU). Mussels were continuously fed with mixtures of the algae *Isochrysis galbana* (T-iso), lyophilized *Phaeodactilum tricornutum* and freshly collected and sieved particles of natural sediment. One group was fed a high organic content diet (80%) dosed at a concentration below the psedofaeces threshold (BP diet: 1-1.5 mm^3/l), whereas the second group was fed a low organic content diet (30%) dosed at a concentration above the pseudofaeces threshold (AP diet: 3-3.5mm^3/l). The particulate organic matter (POM) of BP and AP diet tanks were ≈0.8 and 1.6 mg/l respectively. Diets were continuously pumped to the tanks by peristaltic pumps from concentrated stocks. The concentrations at the tanks were maintained stable by checking frequently with a Coulter Multisizer 3. The shell-lengths and live-weights of individual mussels were measured once every two weeks using 0.05 mm accuracy calipers and a 0.01 mg accuracy balance. Mussels were maintained under these conditions until large inter-individual size differences were found (3 months). After this period, the 24 larger and smaller individuals from each group were selected, representing the percentiles $P_{12.5}$ and $P_{87.5}$ of the size distribution, and denoted as fast (F) and slow (S) growers. Thus, four experimental mussel groups resulted from the combination of the *maintenance condition* (BP and AP) and *growth condition* (F or S):

1. Fast growers selected below the pseudofaeces threshold (F_{BP});
2. Slow growers selected below the pseudofaeces threshold (S_{BP});
3. Fast growers selected above the pseudofaeces threshold (F_{AP}); and
4. Slow growers selected above the pseudofaeces threshold (S_{AP}).

In order to analyze possible differences in the physiological performance of fast and slow growing mussels reared at different maintenance conditions, individuals (n=6) belonging to these 4 groups were used in a series of feeding experiments and the

physiological parameters determining the energy balance were measured. Four experimental diets were used in the feeding experiment, resulting from the combination of 2 food qualities x 2 food concentrations: i) high-quality, low concentration (H_L); ii) high-quality, high concentration (H_H); iii) low-quality, low concentration (L_L); and iv) low-quality, high concentration (L_H). The characteristics of H_L and L_H experimental diets were similar to the BP and AP diets dosed to the mussels during maintenance.

Feeding experiments performed with selected fast (F) and slow (S) growing juveniles.

Characteristics of experimental diets

Experimental diets were made up by mixing cells of the microalgae I*sochrysis galbana* (T-iso) and silt particles. Gravimetric characteristics of each diet were determined every day during experiments of energetic physiology. Water samples of the experimental tanks were filtered onto ashed pre-weighed GF/C glass-fibber filters and subsequently processed to determine total particle matter concentration (TPM: mg/L), inorganic particulate matter (PIM: mg/L) and organic particulate matter (POM: mg/L). Retained salts were rinsed out with a solution of ammonium formate (0.9%). TPM and PIM were estimated as the dry and ash weight increment of the filters respectively. POM was calculated as the difference between TPM and PIM. Organic content (f) was estimated as POM/TPM.

Determination of the physiological parameters

Mussels were fed each experimental diet for one week before starting measuring physiological rates that were determined as described below.

Energy acquisition

Clearance rate (CR: L/h) was measured according to Hildreth and Crisp (1976) as:

$$CR = F^* ((Ci-C_0)/Ci)$$

Where F is the flow rate (L/h), Ci is the particle concentration in the control outflow and C_0 the particle concentration in the outflow of the experimental chamber. Particle concentration was determined with a counter coulter Z1.

A mussel seed was placed in each experimental chamber and the flow rate through the chamber was continuously adjusted to obtain a reduction of 15-30% on the particle concentration compared with the control chamber. Samples of water in the outflow of individual and control chambers were taken every hour during a period of 11-12 hours. Thus, the clearance rate of each individual was calculated as the mean value of 11 to 12 determinations during the whole day.

Filtration rate of total (FR: mg/h) and organic particulate organic matter (OFR: mg/h) were calculated as the product of CR*TPM and CR*POM respectively. When mussels were fed below the pseudofaeces threshold (H_L, H_H and L_L diets), both FR and OFR were equivalent to the ingestion rates of total (IR: mg/h) and organic matter (OIR: mg/h) respectively.

Absorption efficiency (AE: Decimal units) was determined according to Conover (1966), comparing the organic content of the experimental tank water (f) and the organic content of the faeces collected from each of the mussels (h).

AE= (f-h)/(1-f)*h

The resulting *Absorption rate* (AR: mg/h) was computed as the product of OIR and AE.

The mussels produced pseudofaeces when fed the experimental diet L_H, and accordingly absorption rate was calculated as following: the pseudofaeces produced while measuring the clearance rate were collected to calculate the rejection rate of total and organic particulate matter (RR and ORR: mg/h). The proportion of filtered matter that was rejected in pseudofaeces (RP) was computed as RR/FR. The ingestion rate and organic ingestion rate were then computed as the difference between filtration and rejection rates (IR=FR-RR and OIR=OFR-ORR respectively). The preingestive selection efficiency (SE: fraction) was determined according to Kiørboe and Møhlenberg (1981) as SE= 1 - (p /f), where p is the organic content of the pseudofaeces and f the organic content of the food. The faeces produced by each individual during the period of CR measurement were collected to calculate the egestion rate of total and organic matter (ER and OER: mg/h; respectively). The resulting absorption rate (AR) was computed as OIR-OER and absorption efficiency was calculated as AR/OIR.

Metabolic expenditures

After the determination of food acquisition rates, mussels were introduced in individual chambers (150 mL) sealed with LDO oxygen probes connected to oxymeters (HATCH HQ 40d) for the determination of routine oxygen consumption (VO_{2R}: mL O_2/h). The rates of VO_2 were derived from the decrease of the oxygen concentration of the water over time. Water oxygen concentration data were registered every 5-10 minutes until oxygen values decreased by 20-30% of the initial baseline. A control chamber was used to check the stability of the oxygen concentration. Subsequently, mussels were starved for seven days, and the rates of oxygen consumption were measured again to determine the standard oxygen consumption (VO_{2S}: mL O_2/h).

The *routine metabolic rate* (RMR: J/h) and *standard metabolic rate* (SMR: J/h) were estimated from routine and standard oxygen consumption using an oxycaloric coefficient of 20.08 J/mL O_2 (Gnaiger, 1983).

Energy balance

*Scope for growth*_(SFG: J/h) was estimated as the difference between the absorption rate (AR) and the routine metabolic rate (RMR). AR (mg/h) was transformed into energy units (J/h) by using the estimated energy content for *Isochrysis galbana* of 18.75 J/mg reported by White (1987).

Size Standardization

Physiological determinations are expressed in terms of live weight. Clearance rates and oxygen consumptions were standardized to a common live weight of 1 gr. according to the following expression (Bayne and Newell, 1983):

$$Y_{STD} = (1/W_{EXP})^b * Y_{EXP},$$

where Y_{STD} and Y_{EXP} represent, respectively, the standard and experimental physiological rates. W_{EXP} is the experimental weight of the mussel, and b, the power value that scales physiological rates to body weight. The allometric values (b) used for clearance rate and oxygen consumption were 0.58 (Bayne and Hawkins, 1997) and 0.724 (Bayne et al. 1973) respectively.

Gill-surface area (GA: mm^2)

Once the physiological parameters were measured, animals were carefully dissected by cutting abductor muscles. Mussels were placed on a graph paper for setting the scale and a photograph of the internal tissues of each mussel was taken with a digital camera. Gill-surface area was estimated from the photo by ImageJ program. Displayed data correspond to one side of a demibranch. Gill areas were standardized for an equivalent 1 gr live-weight mussel according to the expression:

$$GA_{STD} = (1/W_{EXP})^b * GA_{EXP},$$

where GA_{STD} and GA_{EXP} represent the standardized and experimental gill area, respectively, and W_{EXP} is the experimental live-weight of the mussel. The power function that scales gill area to live-weight was 0.66 (Jones et al. 1992; Vahl, 1973; Hawkins et al. 1992).

Gills were dissected out, inmersed in RNA later and stored at -80 ºC until RNA extraction for molecular analisis (chapter 4)

Statistical analisis

Normality and homogeneity of variances were tested using Shapiro-Wilk and Levene tests, respectively, prior to analysis of the data. Significant differences in growth rates between mussels grown with BP and AP diets were tested by comparing the slope (b) and intercepts (a) using covariance (ANCOVA) procedures described in Zar (2010). The significance level of the effect that the *growth condition* factor (this is, the effect of being fast or slow grower) and the *maintenance condition* factor (this is, having been reared under BP or AP diet), and their interaction might exert on the physiological parameters measured were tested using a two-way factor ANOVA. Differences between groups were analyzed by post-hoc tests, Games Howell or Tukey, according to Levenne test results. Statistical analyses were performed using IBM SPSS Statistics 19.

Results

Growth rates and selection of fast and slow growers

Growth rates (GR: mm/day) of the mussels reared with both diets, below (BP) and above (AP) pseudofaeces threshold respectively, were estimated by adjusting linear regression models to the variations of mean values of shell-lengths with time (days) . The resulting equations were:

BP: GR = 0.139(±0.003) *time + 6.889(±0.088), F = 3,078.4 $p<0.0001$
AP: GR = 0.130(±0.002) *time + 8.524(±0.119), F = 4,386.3, $p<0.0001$

Mussels grew an average of 0.13mm/day in both maintenance conditions. The ANCOVA indicated lack of significant differences in growth rates (Slope test: t=1.77, df=1, 5, $p<0.05$; "intercept" test: t=-34.54, df=1, 5, $p>0.05$). Inter-individual differences in the growth rate of mussels in each rearing condition were so apparent that a rearing period of 75 days was long enough to easily select fast and slow growers from each condition. The live-weight of F individuals was 2.5 fold higher than that from S individuals, and the shell-length was 45% larger (Table 1.1).

Table 1.1. Shell-length (mm) and live weight (g) (mean ± SD) of mussels. Initial values upon arrival to the laboratory (n= 200) and values corresponding to mussels selected as fast and slow growers (n=24).

	Initial	F_{BP}	S_{BP}	Initial	F_{AP}	S_{AP}
Length (mm)	9.6 ± 0.35	21.2 ± 0.70	13.9 ± 1.15	10.7 ± 0.33	21.9 ± 0.6	15.4 ± 1.01
Weight (g)	0.15 ± 0.02	0.95 ± 0.12	0.35 ± 0.05	0.19 ± 0.03	1.04 ± 0.09	0.47 ± 0.09

Feeding experiments with selected fast and slow growers

The characteristics of the four diets (H_L, H_H, L_L and L_H) used in feeding experiments are shown in Table 1.2. The particulate organic matter (POM) was around 0.4 mg/L for the diets dosed at low concentration and around 1.4 mg/L for the diets supplied at high concentration. The organic content of high and low quality diets was approximately 0.8 and 0.4 respectively.

Table 1.2. Characteristics of the experimental diets. TPM: total particulate matter; PIM: particulate inorganic matter; POM: particulate organic matter and OC: organic content. H_L: high-quality low concentration; H_H: high-quality high concentration, L_L: low-quality low concentration, L_H: low-quality high concentration.

Diet	TPM (mg/L)	PIM (mg/L)	POM (mg/L)	OC (fraction)
H_L	0.58 ± 0.05	0.10 ± 0.04	0.49 ± 0.01	0.83 ± 0.06
H_H	1.47 ± 0.20	0.30 ± 0.07	1.17 ± 0.12	0.80 ± 0.03
L_L	0.71 ± 0.16	0.37 ± 0.14	0.34 ± 0.03	0.49 ± 0.07
L_H	4.57 ± 0.43	2.89 ± 0.71	1.68 ± 0.33	0.37 ± 0.10

Feeding and preingestive processes.

Clearance rates measured for the selected four groups of mussels (F_{BP}, S_{BP}, F_{AP} and S_{AP}) when fed each one of the experimental diets have been plotted as a function of POM in Figure 1.1. Three trends may be highlighted: a) Clearance rates exponentially decreased with increasing food concentration, irrespective of inter-group differences. b) CR values corresponding to mussel groups sharing *growth condition* characteristics (fast growers (F_{BP} and F_{AP}) vs slow growers (S_{BP} and S_{AP})) were more similar between them than values corresponding to mussels groups sharing *maintenance condition* (BP vs AP). c) Fast growing mussels (full symbols) displayed systematically higher clearance rates than slow growing mussels (empty symbols), for the whole range of tested POM.

The two-factor analysis showed that the *growth condition* factor was found to exert a highly significant effect in the CR of the four experimental conditions (Table 1.4), while no significant effect of the *maintenance condition* was observed. A significant effect of the interaction term was recorded only for the H_L diet, accounting for the fact that differences in the CRs between fast and slow growing mussels that were reared with diets above pseudofaeces threshold (AP) were very small and not even significant.

Table 1.3. Physiological parameters (mean ± SD) measured in mussels during feeding experiments: i) high-quality low concentration (H_L), ii) high-quality high concentration (H_H), iii) low-quality low concentration (L_L) and iv) low-quality high concentration (L_H). Mussel groups: i) fast grower below pseudofaeces threshold (F_{BP}), ii) slow grower below pseudofaeces threshold (S_{BP}), iii) fast grower above pseudofaeces threshold (F_{AP}), iv) slow grower above pseudofaeces threshold (S_{AP}). Physiological parameters: CR: clearance rate (L/h), SE: selection efficiency (fraction), RP: rejection proportion, OIR: organic ingestion rate (mg/h), AE: absorption efficiency (fraction), AR: absorption rate (mg/h), VO_{2R}: routine oxygen consumption (mL/h), VO_{2S}: standard oxygen consumption (mL/h) and SFG: scope for growth (J/h). Letters indicate statistical aggrupation of growth groups per parameter according to the corresponding post hoc test.

	F_{BP}	S_{BP}	F_{AP}	S_{AP}
H_L diet	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
CR (L/h)	0.66 ± 0.10 ^{a}	0.30 ± 0.14 ^{b}	0.48 ± 0.17 $^{a.b}$	0.40 ± 0.14 ^{b}
OIR (mg/h)	0.32 ± 0.05	0.15 ± 0.07	0.24 ± 0.08	0.19 ± 0.07
AE (fraction)	0.71 ± 0.07 ^{a}	0.78 ± 0.08 $^{a.b}$	0.72 ± 0.03 $^{a.b}$	0.82 ± 0.06 ^{b}
AR (mg/h)	0.23 ± 0.05 ^{a}	0.12 ± 0.05 ^{b}	0.17 ± 0.06 $^{a.b}$	0.16 ± 0.06 $^{a.b}$
VO_{2R} (mL/h)	0.065 ± 0.019^{a}	0.053 ± 0.014 ^{a}	0.060 ± 0.007 ^{a}	0.078 ± 0.029 ^{a}
VO_{2S} (mL/h)	0.037 ± 0.008 ^{a}	0.043 ± 0.014 ^{a}	0.035 ± 0.016 ^{a}	0.029 ± 0.014 ^{a}
SFG (J/h)	3.03 ± 0.86^{a}	1.11 ± 1.18^{b}	2.01 ± 1.17$^{a.b}$	1.33 ± 1.06$^{a.b}$
H_H diet				
CR (L/h)	0.39 ± 0.08 ^{a}	0.22 ± 0.05 ^{b}	0.39 ± 0.09 ^{a}	0.20 ± 0.09 ^{b}
OIR (mg/h)	0.47 ± 0.11	0.27 ± 0.14	0.48 ± 0.11	0.24 ± 0.11
AE (fraction)	0.55 ± 0.08 ^{a}	0.68 ± 0.04 ^{b}	0.62 ± 0.03 $^{a.b}$	0.67 ± 0.08 ^{b}
AR (mg/h)	0.26 ± 0.08 ^{a}	0.18 ± 0.08 ^{a}	0.30 ± 0.08 ^{a}	0.16 ± 0.08 ^{a}
VO_{2R} (mL/h)	0.075 ± 0.018^{a}	0.067 ± 0.017 ^{a}	0.068 ± 0.023 ^{a}	0.043 ± 0.03 ^{a}
VO_{2S} (mL/h)	0.045 ± 0.011 ^{a}	0.052 ± 0.007 ^{a}	0.047 ± 0.02 ^{a}	0.032 ± 0.017 ^{a}
SFG (J/h)	3.37 ± 1.25 ^{a}	2.65 ± 1.62 ^{a}	4.20 ± 1.05 ^{a}	2.62 ± 1.68 ^{a}
L_L diet				
CR (L/h)	0.85 ± 0.12 $^{a.c}$	0.46 ± 0.14 ^{b}	1.05 ± 0.28 ^{c}	0.51 ± 0.27 $^{a.b}$
OIR (mg/h)	0.29 ± 0.04	0.16 ± 0.05	0.36 ± 0.10	0.17 ± 0.09
AE (fraction)	0.76 ± 0.03 $^{a.b}$	0.70 ± 0.08 ^{b}	0.79 ± 0.01 ^{a}	0.76 ± 0.03 $^{a.b}$
AR (mg/h)	0.22 ± 0.03 $^{a.c}$	0.11 ± 0.04 ^{b}	0.28 ± 0.08 ^{c}	0.13 ± 0.07 $^{a.b}$
VO_{2R} (mL/h)	0.067 ± 0.011^{a}	0.064 ± 0.017 ^{a}	0.057 ± 0.009 ^{a}	0.052 ± 0.012 ^{a}
VO_{2S} (mL/h)	0.038 ± 0.011 ^{a}	0.034 ± 0.013 ^{a}	0.033 ± 0.008 ^{a}	0.043 ± 0.016 ^{a}
SFG (J/h)	2.76 ± 0.60 $^{a.b}$	0.80 ± 0.84 ^{c}	4.13 ± 1.57 ^{a}	1.45 ± 1.31 $^{b.c}$
L_H diet				
CR (L/h)	0.35 ± 0.09^{a}	0.20 ± 0.04^{b}	0.29 ± 0.10$^{a.b}$	0.20 ± 0.04^{b}
RP(fraction)	0.55 ± 0.12^{a}	0.47 ± 0.11$^{a.b}$	0.55 ± 0.06^{a}	0.41 ± 0.10^{b}
SE(fraction)	0.39 ± 0.06$^{a.b}$	0.35 ± 0.05^{b}	0.44 ± 0.06^{a}	0.38 ± 0.05$^{a.b}$
OIR (mg/h)	0.35 ± 0.07^{a}	0.23 ± 0.06^{b}	0.34 ± 0.13$^{a.b}$	0.25 ± 0.04^{b}
AE(fraction)	0.68 ± 0.04$^{a.b}$	0.61 ± 0.06^{b}	0.72 ± 0.04^{a}	0.66 ± 0.05^{b}
AR(mg/h)	0.24 ± 0.06^{a}	0.14 ± 0.03^{b}	0.25 ± 0.10$^{a.b}$	0.16 ± 0.03^{b}
VO_{2R} (mL/h)	0.072 ± 0.013^{a}	0.062 ± 0.018^{a}	0.052 ± 0.016^{a}	0.058 ± 0.022^{a}
VO_{2S} (mL/h)	0.043 ± 0.009^{a}	0.035 ± 0.012^{a}	0.031 ± 0.010^{a}	0.041 ± 0.009^{a}
SFG (J/h)	3.05 ± 1.23^{a}	1.38 ± 0.77^{b}	3.55 ± 1.87^{a}	1.91 ± 0.72$^{a.b}$

Table 1.4. P values of two-way factor ANOVAs testing significant effects of *growth condition* (F or S) and *maintenance condition* (BP or AP) on physiological parameters of mussels when fed the four experimental diets (H_L, H_H, L_L and L_H) of Treatment I.

	H_L	H_H	L_L	L_H
CR				
Maintenance condition	0.511	0.696	0.170	0.322
Growth condition	**0.001**	**0.002**	**<0.001**	**<0.001**
Interaction	**0.026**	0.623	0.430	0.270
RP				
Maintenance condition	n.d.	n.d.	n.d.	0.380
Growth condition				**0.006**
Interaction				0.375
SE				
Maintenance condition	n.d.	n.d.	n.d.	**0.037**
Growth condition				**0.026**
Interaction				0.588
OIR				
Maintenance condition	0.508	0.701	0.168	0.898
Growth condition	**0.001**	**0.002**	**<0.001**	**0.002**
Interaction	**0.026**	0.624	0.430	0.543
AE				
Maintenance condition	0.313	0.269	**0.024**	**0.015**
Growth condition	**0.003**	**0.002**	**0.027**	**0.001**
Interaction	0.648	0.129	0.543	0.784
AR				
Maintenance condition	0.763	0.583	0.095	0.484
Growth condition	**0.013**	**0.010**	**<0.001**	**<0.001**
Interaction	**0.035**	0.595	0.402	0.632
VO_{2R}				
Maintenance condition	0.190	0.111	**0.044**	0.079
Growth condition	0.728	0.083	0.445	0.739
Interaction	0.065	0.379	0.789	0.239
VO_{2S}				
Maintenance condition	0.158	0.147	0.623	0.333
Growth condition	0.939	0.439	0.578	0.760
Interaction	0.273	0.080	0.168	**0.024**
SFG				
Maintenance condition	0.387	0.502	**0.042**	0.269
Growth condition	**0.009**	0.063	**<0.001**	**0.001**
Interaction	0.185	0.472	0.448	0.970

Mussels from the selected four groups were found to reduce clearance rate but also to produce pseudofaeces when fed experimental diet $L_{H,}$ as a way to limit the ingestion rate. Values corresponding to the proportion of filtered matter that was rejected and the selection efficiency of the pseudofaeces production process recorded for each mussel group are shown in Table 1.3. Fast growers showed significantly higher rejection rates both in total (this would be in good correspondence with their higher filtration rates) and relative terms: the rejected fraction accounted for 55% and 44% of

filtration rate in F and S mussels, respectively. A significant effect of the *growth condition* factor was confirmed by the results of the two factor ANOVA shown in Table 1.4. The post-hoc comparison showed that whereas differences found between BP mussels did not attain the required level, significantly different rejected proportions were found when F_{AP} and S_{AP} mussels groups were compared.

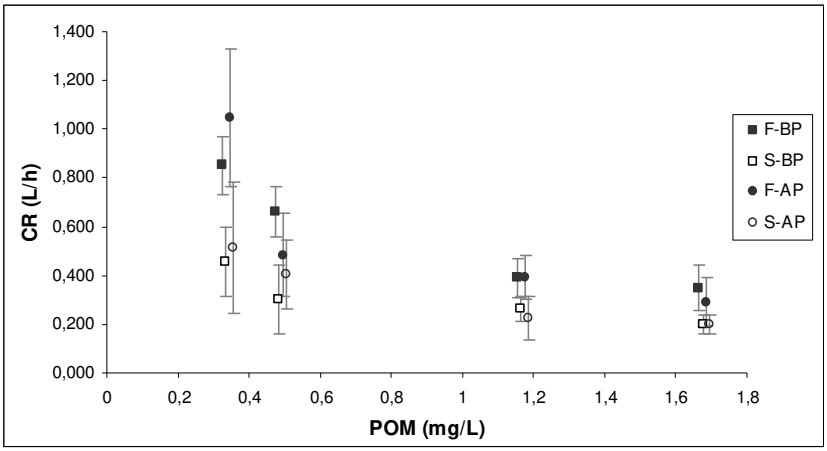

Figure 1.1. Clearance rate (L/h) of $F_{BP,}$ S_{BP} F_{AP} and S_{AP} mussels as a function of particulate organic matter (POM: mg/L).

Regarding the capacity to selectively reject inorganic matter and preferentially ingest organic material, although differences in the index reported for each mussel group were small, both factors *growth condition* and *maintenance condition* (Table 1.4) were found to exert a significant effect on the selection efficiency (SE): F individuals were better selecting organic matter than S individuals, and mussels reared in AP conditions were more efficient than their BP counterparts.

Irrespective of maintenance conditions, and in correspondence with the recorded differences in CR for diets H_L, H_H and L_L, higher rates of organic matter filtration (OFR) and ingestion (OIR) were recorded for F mussels than for S mussels; in the case of diet L_H the production of pseudofaeces caused that total and organic ingestion rates were reduced in comparison with L_L diet (see Figure 1.2), but as a result of the higher selection efficiencies and greater proportion of rejection in F mussels, significant differences in organic ingestion rate between F and S mussels remained (Figure 1.2, Table 1.3).

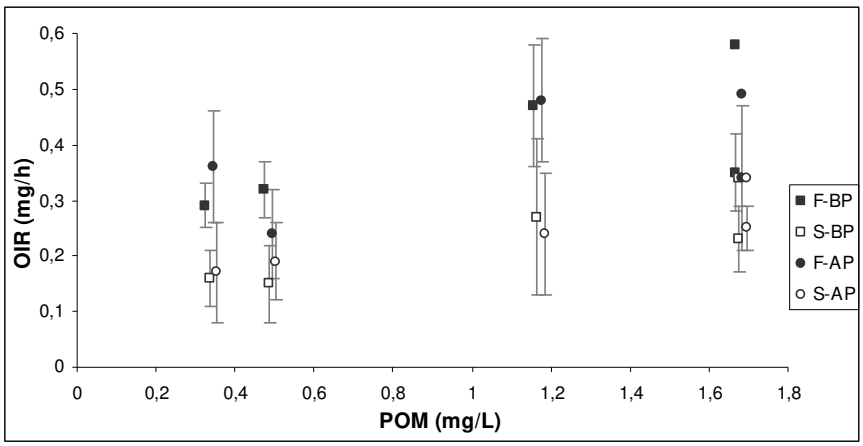

Figure 1.2. Organic ingestion rate (mg/h) of $F_{BP,}$ S_{BP} F_{AP} and S_{AP} mussels as a function of particulated organic matter (POM:mg/L). In the POM value representing L_H diet, the organic filtration rates (mg/h) of each mussel group has been added (without SD, for clarity).

Digestion and absorption processes.

Figure 1.3 shows the mean values of absorption efficiency of food measured for each one of the four selected mussel groups (F_{BP}, S_{BP}, F_{AP} and $S_{AP)}$ when fed each one of the four experimental diets, as a function of ingestion rates of organic matter recorded for them. A trade-off between both parameters was observed: as a rule, mussels with higher organic ingestion rates were found to absorb organic matter from the food with a lower efficiency than mussels ingesting particles at lower rates. A linear regression analyse brought to light the existence of a significant ($p=0.024$) negative correlation between these two parameters.

On the other hand, when addressing differences found between mussel groups fed the different experimental diets, some interesting results came out: after testing the effect of the *growth condition* (F vs S) and *maintenance condition* (BP vs AP) factors on the absorption efficiency (see the two way ANOVA results in Table 1.4), *growth condition* was found to exert significant effect on AE in the four feeding conditions. The interpretation of this result is far for being direct, however, since whereas higher values of absorption efficiency were reported for slow growing mussels fed the high quality diets than for their fast growing counterparts, quite the opposite was found for mussels fed the low quality diets (Table 1.3). So, the general rule above mentioned (Figure 1.3) was applicable to inter/group differences observed between mussels fed high quality

diets, but not for inter/group differences observed between mussels fed low quality diets, where faster feeding mussels were capable of absorbing organic matter more efficiently that slow feeding mussels.

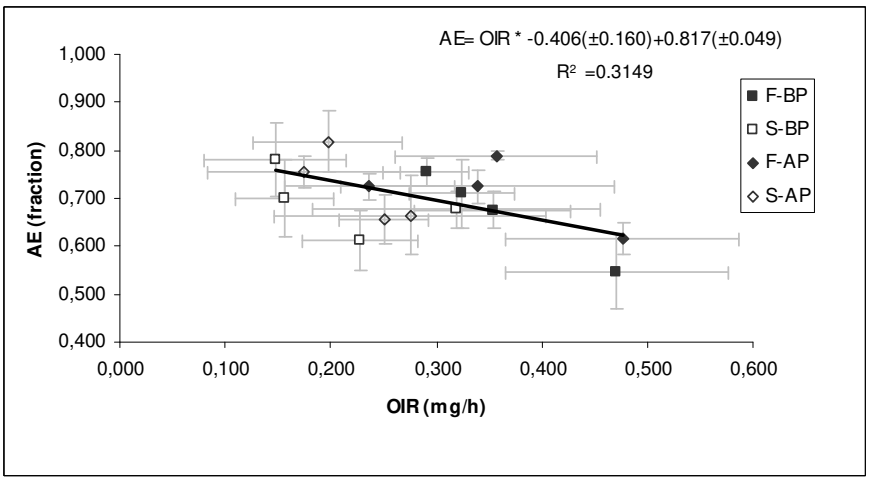

Figure 1.3. Absorption efficiency of the four mussels groups ($F_{BP,}$ S_{BP} F_{AP} and S_{AP}) as a function of organic ingestion rate (mg/h).

Following with the special features of mussels fed experimental diets of low organic content ($L_L$and L_H diets), besides *growth condition*, also *maintenance condition* was found to affect significantly the absorption efficiency: mussels reared under conditions promoting continuous pseudofaeces production (AP) absorbed ingested organic matter with an efficiency that was slightly but significantly higher than the efficiency reported for mussels reared under conditions which never lead to reject particles retained at their gills (BP).

The combination of organic ingestion rates and absorption efficiencies determines the rates of absorption (AR); those rates have been plotted in Figure 1.4 as a function of particulate organic matter (POM). It is clear that some of the largest differences observed in Figure 1.2 when OIR were considered, were lessened as a result of the corrective effect exerted by absorption efficiency. Irrespective of the maintenance condition, fast growing mussels (full symbols) attained higher rates of food absorption than slow growing mussels (empty symbols) in the whole range of food concentration. Significant effects of *growth condition* and *maintenance condition* on absorption rate were tested by the two-factor ANOVA shown in Table 1.4. The pattern resembles the

one previously explained for CR i) *growth condition* exerted a significant effect on AR for all the four experimental diets, ii) no significant effect of the *maintenance condition* was observed, and iii) the interaction term was significant only for mussels fed H_L diet; as before, this accounted for the fact that almost identical absorption rates were recorded for F and S mussels reared under AP conditions.

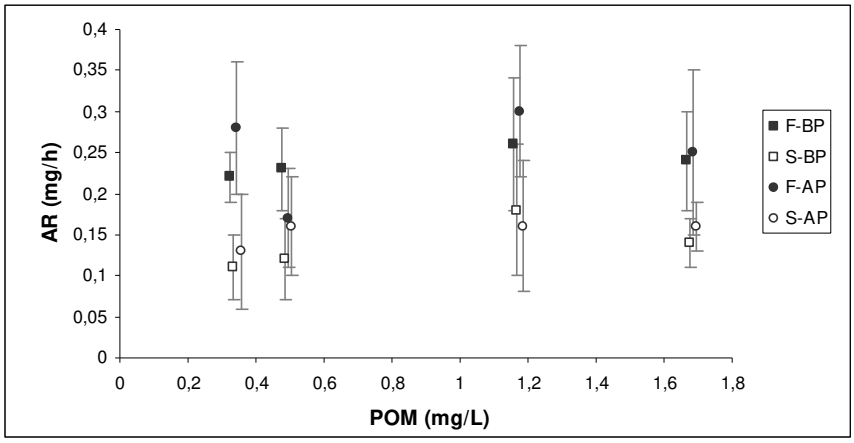

Figure 1.4. Absorption rate (mg/h) of the four mussel groups ($F_{BP,}$ S_{BP} F_{AP} and S_{AP}) as a function of particulated organic matter (POM: mg/L).

Routine and standard metabolic rates

Mean values of the Routine oxygen consumption (VO_{2R}) values obtained for the four selected mussel groups when fed each one of the experimental diets were plotted as a function of POM in Figure 1.5. No clear trend was observed from these data, nor was it observed when analysing standard oxygen consumption (VO_{2S}) measured to each of the four groups of mussels after seven days of fasting (see values in Table 1.3)

To analyse the potential effect of the *growth condition* and *maintenance condition* factors on the routine and standard oxygen consumptions, a two-factor ANOVA was performed with the values recorded with mussels fed each experimental diet (Table 1.4). *Growth condition* did not exert any significant effect on standard or routine oxygen consumption in any of the experimental diets. *Maintenance condition* exerted a slightly significant effect ($p=0.044$) only on the VO_{2R} of mussels fed L_L diet. This factor accounted for the fact that mussels reared feeding the diet AP displayed lower routine oxygen consumptions than those mussels that were reared with BP diet.

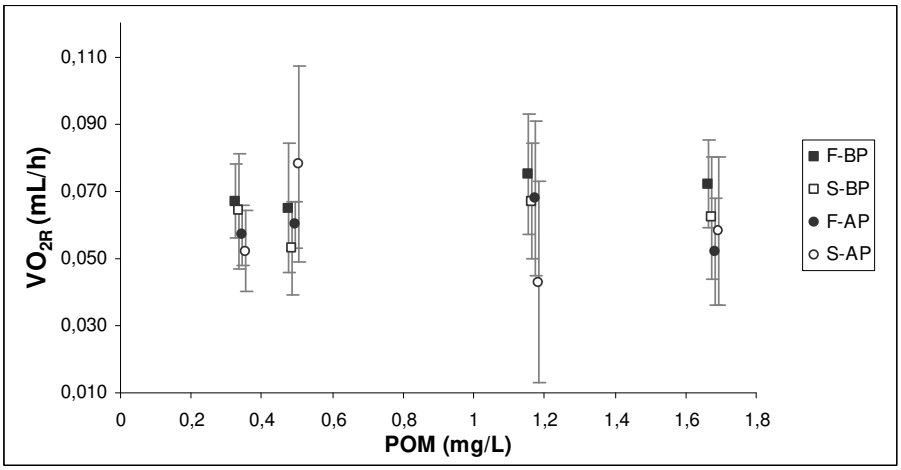

Figure 1.5. Routine oxygen consumption (mL/h) of the four mussel groups ($F_{BP,}$ S_{BP} F_{AP} and S_{AP}) as a function of particulated organic matter (POM: mg/L).

Energy balance

The SFG of mussels from the four experimental groups (F_{BP}, S_{BP}, F_{AP} and S_{AP}) fed the four experimental diets, calculated as the difference between the rate of energy absorbed (Figure 1.4) and the rate of energy loss (Figure 1.5) through the respiration, have been plotted as a function of POM in Figure 1.6. Since values of routine oxygen consumption kept quite constant, differences in SFG values resemble those observed in AR values. The figure evidences that: a) Mussels were able to keep values of SFG quite constant through the experimental conditions, especially those selected as fast growers. b) Values for the balance between energy inputs and outputs corresponding to mussels groups sharing *growth condition* characteristics (fast growers (F_{BP} and F_{AP}) vs slow growers (S_{BP} and S_{AP})) were more similar between them than values corresponding to mussels groups sharing *maintenance condition* (BP vs AP). c) Fast growing mussels (full symbols) attained higher SFG values than slow growing mussels (empty symbols) for the whole range of POM.

The two-factor analysis of variance performed in order to analyse the effect of *maintenance condition* and *growth condition* on the SFG of mussels (Table 1.4) showed that *growth condition* exerted a significant effect in the SFG of mussels fed each one of the experimental diets except H_H diet (the p value, p= 0.063 was closed to statistical significance): the *maintenance condition* factor exerted only a minor effect, a slightly

significant effect (p= 0.042) in mussels fed L_L diet that was derived from the above mentioned differential response observed in the RMR of mussels reared fed AP diets, which resulted in higher SFG values measured for mussels reared AP diets than those reared with BP diets.

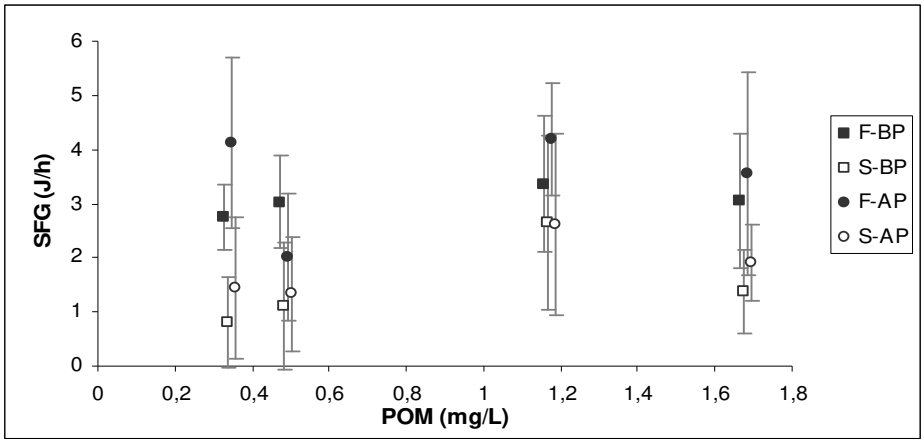

Figure 1.6. Scope for growth (J/h) of the four mussel groups ($F_{BP,}$ S_{BP} F_{AP} and S_{AP}) as a function of particulated organic matter (POM: mg/L).

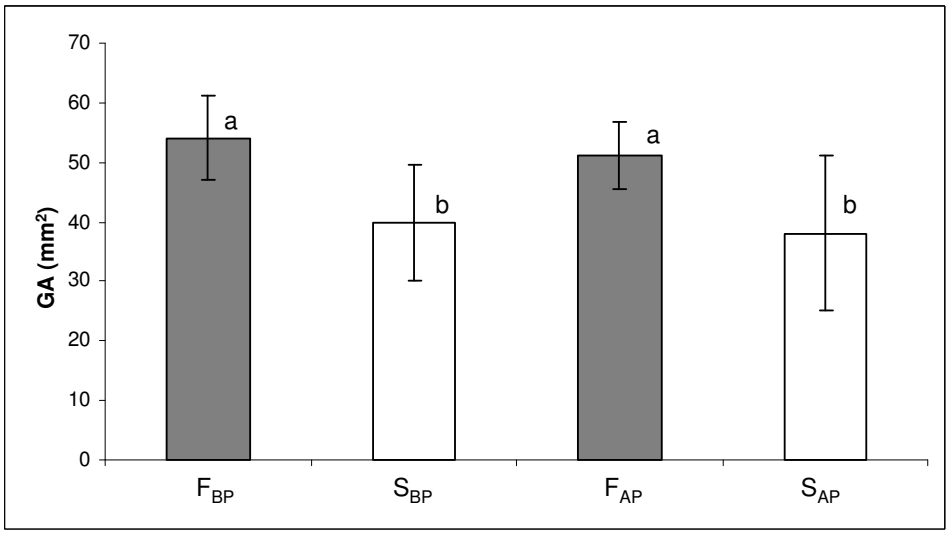

Figure 1.7. Gill surface-area (mean ± SD) of mussels. Non-significant differences are marked with the same letter.

Gill surface-area of selected F and S mussels

Gill area mean values ± SD and the statistical comparison between groups is shown in Figure 1.7. Irrespective of the *maintenance condition*, gill surface area of F mussels was significantly higher than that of S mussels. The Two-way factor ANOVA performed to test the effect of *growth condition* and *maintenance condition* on the gill surface area (cm2) of the mussels (Table 1.5), confirmed that only the *growth condition* factor exerted a significant effect: F mussels had notably higher gill areas than S mussels; neither the *maintenance condition* nor the interaction term g*rowth condition* * *maintenance condition* arose as significant factors.

Table 1.5. Two-way factor ANOVA testing the effect of *growth condition* (F vs. S) and *maintenance condition* (BP vs. AP) on gill surface area (cm^2) of the mussels.

Source of variation	DF	SS	MS	F	P
Growth condition	1	0.175	0.175	26148	**<0.001**
Maintenance condition	1	0.000	0.000	0.004	0.160
Interaction	1	0.005	0.005	0.692	0.694
Residual	89	0.595	0.007		

Discussion

The physiological components that determine interindividual growth differences between individuals living under identical environmental conditions may be modulated by differences in i) food adquisition and assimilation ii) in the allocation of energy for maintenance, growth or reproduction or iii) in the metabolic costs of growth, (Bayne 1999), or by combinations of some or all those differences. The scope for growth provides a useful tool for integrating basic physiological processes as a balance of energy that results in a good proxy of the energy available for growth (shell and/or somatic growth and/or reproduction) in bivalves (see review by Bayne et al 1985). In this study, the selection of fast (F) and slow (S) growing mussels in the two maintenance conditions, one above (AP) and the other below the pseudofaeces threshold (BP), was based on shell size and live weight measurements of the individuals, thus, on experimentally measured growth rates. Once selected as fast and slow growers, the scope for growth values recorded in feeding experiments were used to estimate the

growth capacities corresponding to each mussel group under several experimental conditions. Thus, the potential effect of variables such as the *growth condition* (mussels being F or S growers) or the *maintenance condition* (AP or BP) on the physiological parameters determining the energy balance and on the balance itself (SFG) were tested.

For an accurate experimental design, election of the rearing conditions had to fulfill two requirements: i) to be different enough to potentially exert a differential effect upon the physiological features that could better turn out into higher growth rates, and ii) to promote levels of differentiation between F and S individuals that should not be very dissimilar, in order to be able to run experiments with all mussel groups at a time without great size differences among them. As it can be checked in Table 1.1, mussels selected as fast growers in both maintenance conditions (F_{AP} and F_{BP}) showed similar growth rates (0.146 ± 0.009 and 0.144± 0.007 mm/day) that were more than twice the growth rate of their slow growing counterparts (S_{AP} and S_{BP}) that, in turn, were very similar between them (0.055 ± 0.015 and 0.060 ± 0.013 mm/day). Correspondingly, the SFGs recorded with F_{AP} mussels (3.5 to 4 J/h) with L_L and L_H diets (i.e. diets of a similar quality to AP maintenance condition) were similar to the SFGs of F_{BP} mussels (3 to 3.4 J/H) fed H_L and H_H (i.e. diets of a similar quality to BP maintenance condition). The same was found for S_{AP} mussels fed L_L and L_H (1.5-1.9 J/h) as compared with S_{BP} specimens fed H_L and H_H (1.1-2.65 J/h). We can not conclude from this comparison that SFG values matched exactly the real growth rates, however, this level of coincidence made us feel confident about the use of the scope for growth as a good indicator of the growing capacity of mussels under any tested stable condition.

One aim of this study was to confirm or otherwise refute the conclusions of earlier experiments on the physiological basis for faster rates of growth in mussels selected under different rearing conditions (Tamayo et al. 2016; Prieto et al. 2018-chapter 2). Inter-individual endogenous (genetic) differences in the capacity to produce pseudofaeces to limit ingestion rate, and in the ability to select organic particles, could be *a priori* considered a differential trait potentially contributing to size differentiation under food regimes above the threshold for pseudofaeces production. Quite a lot of studies focusing on the physiological ecology of bivalves have demonstrated that selective ingestion of high-quality organic material is a key component of feeding physiology and scope for growth of many species of bivalves (see review by Ward and Shumway 2004) inhabiting turbid waters. In contrast, with the exception of the studies

of Bayne et al. (1999a) and Bayne (2004), performed with the pacific oyster and the Sidney rock oyster respectively, there is no information about the effect that intraspecific differences in the rates and efficiencies of the preingestive processes of the food could have in the interindividual differences in growth rates of bivalves. Moreover, it was not until now, that a complex experimental approach was conducted to ascertain if a long-term acclimation of specimens to nutritional conditions resembling those corresponding to turbid waters (i.e promoting continuous production of pseudofaeces) might cause interindividual differences in growth rate specifically linked to differential efficiencies in the preingestive handling of heavy seston loads. Additionally we explored the extent to which the specificities in the physiological profiles driven by the rearing nutritional conditions prevailed or affected the well-known phenotypic plasticity of mussels to respond to changes in their nutritional environment.

Based on that approach, we analyzed the effect that two main factors exerted on the physiological parameters involved in the energy balance of mussels selected as fast (F) or slow growers (S) after being reared for 3 months half of them under feeding conditions above the pseudofeces production threshold (AP) and the other half below that threshold (BP), when exposed to four different feeding experimental environments: i) the first one named *growth condition* factor was representative of the endogenous component responsible for interindividual differences between F and S mussels reared under the same conditions, and ii) the second one named *maintenance condition* factor, representing rearing nurture conditions prevailing until F and S mussels differentiated, which was controlled by keeping nutritional conditions below (BP) or above (AP) the pseudofaeces producing threshold..

Obtained results indicate that, contrary to what we expected and formulated as a hypothesis in the introduction, tested nurturing conditions prevailing during the rearing period did not cause any effect on the differential innate physiological capacities underlying the growth capacity, since irrespective of the past feeding history (*maintenance condition*) during the size-differentiation period, mussels selected as fast or slow growers under the two maintenance conditions shared common physiological features. This conclusion can readily be obtained from the results of the ANOVA shown in Table 1.4: contrary to what happened with the *growth condition* factor, the effect of *maintenance condition* did not reach the required significance level ($p<0.05$) for any of the physiological rates governing energy acquisition (CR, RR, OIR, AR) and

expenditure (VO_{2R} and VO_{2S}) in the four groups of mussels tested when exposed to four experimental diets. To be precise, there was one experimental condition where we found that the maintenance condition exerted a barely significant effect (p= 0.044) on the routine oxygen consumption (Table 1.4). This accounted for the fact that when we measured oxygen consumption in mussels feeding L_L diet, we recorded lower values for mussels reared at AP condition (0.052-0.057 mLO_2/h) than for mussels reared at BP condition (0.064-0.067 mLO_2/h) (values taken from Table 1.3). However, considering that the significance level was at the limit, that such a difference was not observed for any other experimental condition, and that the post-hoc analysis indicated that VO_2 were not different among groups (see Table 1.3), we tend to think that this significant effect was exceptional and does not reflect a true significant difference in the metabolic efficiency that stems from the rearing condition. On the other hand, we did found that the *maintenance condition* factor exerted a significant effect on some relative indexes such as the absorption efficiency and the selection efficiency. Regarding the selection efficiency, there was only one experimental condition (L_H diet) where we could test the effect of the *maintenance condition* (p=0.037) and the *growth condition* (p=0.026), and both factors emerged as significant, indicating that as a general rule fast growers selected organic matter for ingestion more efficiently than slow growers, but also that overall, mussels "trained" in pseudofaeces production, were more efficient at rejecting inorganic matter than their counterparts which never before produced pseudofaeces. Post-hoc analysis (Table 1.3) indicated that "pure" significant differences were only found for SE values corresponding to S_{BP} (35%) and F_{AP} (44%) mussel groups whereas values from F_{BP} (39%) and S_{AP} (38%) were in between. In a similar experiment performed by our team using similar rearing and experimental conditions, but with mussels submitted to a tidal emersion regime of 8h hours out of 24 each day (Prieto et al. 2018 – chapter 2), significant differences in SE between F and S mussel were also found without significant effect of the *maintenance condition*. Thus, we tend to give credence to the idea that *growth condition* exerts a significant effect upon preingestive selection efficiency. The most remarkable feature in the differences in SE between F and S was that such differences were not restricted to mussels reared under high particle concentration (i.e., under conditions compelling pseudofaeces production) but were also present in mussels grown with low particle concentrations (i.e., under conditions in which pseudofaeces were absent). Thus, it seems that a higher ability for pre-ingestive

selection is an inherent feature of fast-growing mussels, rather than being an effect promoted by long-term acclimation to the diet.

Concerning absorption efficiency, the *maintenance condition* appeared as a significantly affecting factor when the selected four mussel groups were fed diets of low organic content, independently of the dosing level or quantity of food. This happened because both fast and slow growing AP mussels digested and absorbed ingested matter more efficiently than their BP counterparts. As explained before, AP mussels were more efficient at selecting organic matter over the bulk for preferential ingestion, and as a result, they were more capable of enriching the organic content of the ingested fraction. As it has been previously stated, in bivalves, the absorption efficiency is positively dependent on the quality of the ingested food; the slightly higher efficiency performance of the pallial organs of AP mussels over the BP mussels could have turned to improve the absorptive capacity of these individuals, although that some kind of additional digestive adaptation occurred during the rearing period can not be discarded.

Unlike the effect of the *maintenance condition* above commented, the *growth condition* factor came out once and again as a key factor explaining differential responses observed among fast growing and slow growing mussels. This was so when the energy acquisition processes were considered; however, we did not observed any effect on the routine metabolic rate and neither on the standard oxygen consumption (see ANOVA results at Table 1.4). The absence of an effect on the VO_{2S} may be understand quite easily, since after one week of starving only basic processes of cell maintenance would go on, and those could be quite similar (see values in Table 1.3) irrespective of the rearing conditions or even irrespective of the growing rate if differential growing rates were not based on differences in the energy allocation processes. However, the lack of any effect on the routine energy expenditure was unexpected (Figure 1.5). Routine metabolic rate has been shown several times to be a rising function of absorption rate in bivalves (Thompson and Bayne, 1974; Bayne et al. 1989; Bayne 1999; Babarro et al. 2000). Metabolic cost associated with the processes of feeding and absorption represents 15-30% of the absorbed ration (Widdows and Hawkins, 1989; Bayne et al. 1989). Our results did not show any significantly higher metabolic rate with increasing AR: in fact, very similar metabolic rates were measured in F and S mussels in spite that fast growers achieved absorption rates that doubled the ones of slow growers. This means that, in addition to higher capacity to acquire and

process food, F mussels seem to have a higher metabolic efficiency and/or lower costs of growth, which also constitutes a feature promoting faster growing. The coupling of high feeding rates with low costs of growth in faster growing oysters has been previously reported (Bayne et al. 1999a, b; Bayne 2000; Pernet et al. 2008; Pace et al. 2006; Toro et al. 1996; Toro and Vergara 1998), and inter-individual differences in metabolic efficiency have been typically explained as the consequence of the existence of endogenous differences in the efficiency of protein metabolism (Hawkins et al. 1986; Bayne and Hawkins, 1997; Hulbert and Else, 2000). There has been also quite a lot of work done concerning the genetic or transcriptomic basis of the metabolic based differences in growth rate observed in bivalves; Hedgecock et al. (2007) and Meyer and Manahan (2010) showed that several genes are differentially expressed in fast versus slow growing larvae of oyster larvae, and differences were basically found to affect genes involved in protein metabolism. Further analyses are needed in order to link differential gene expression among growth categories to phenotypic variation in physiological components of growth, and specially to understand differences in metabolic efficiency. In this sense, although not applied to bivalves, some advances recently reported by using metabolomics to characterize the metabolic differences related to growth variation in *Haliotis midae* (Venter et al. 2018) are welcome, since they will greatly contribute to clarify this fundamental aspect of the growth.

Regarding the physiological parameters involved in incorporating energy, obtained experimental data strongly supported an increased energy acquisition model (*sensu* Bayne, 1999) for explaining differences in the growing rate between fast and slow growing mussels, irrespective of the rearing condition; in brief: faster growth derived from faster rates of food consumption and absorption. Results from the ANOVA reported in Table 1.4 clearly show that whatever the endogenous component that during the rearing period determined the size differentiation between fast and slow growers, it resulted in a better physiological performance that persisted and conferred fast growing mussels selected at both maintenance conditions a greater capacity to ingest and absorb food energy (see Figures 1.1 to 1.5). Mussels selected as fast growers, irrespective of the rearing condition, showed significantly higher clearance rates than slow growers (Figure 1.1) through all the experimental diets, (e.g. in H_H diet, the CR values for F_{BP} and F_{AP} mussels were around 0.39±0.09 L/h whereas for S_{BP} and S_{AP} was about 0.21± 0.07 L/h respectively). This general pattern was also observed for the

L_H diet, but at this particular experimental condition the high concentration of low quality particle in the water column imposed that additional preingestive processes had to operate in all the experimental mussel groups studied: fast growing mussels were found to reject a larger proportion of filtered matter, and to better select food items, irrespective of the nurturing conditions. As a result, fast growing mussels were found to show higher ingestion organic rates, this is, incorporated more energy per time unit at their digestive system.

Absorption efficiencies, however, were not always higher for F mussels as a result of the well-known trade-off between the time it takes the food to pass the digestive system. In continuous feeders such as bivalves, ingestion rate modulates gut passage time (GPT: available time for the digestive system for food digestion and absorption), which, in turn, is the main factor determining absorption efficiency: reduction of GPT with rising ingestion rates and decreased AE with shorter GPT are common features in the feeding physiology of bivalves (Bayne et al. 1989; Navarro et al. 1994), and was also observed under the feeding conditions tested in this experiment. In fact, equation shown in Figure 1.3 shows that a 31% of the variation observed in AE was caused by the reduction in GPT derived from rising ingestion rates, and underlines the importance of regulating the rates of ingestion as a way to optimize absorptive processes.

Although higher organic ingestion rates recorded in F mussels sometimes resulted in slightly lower absorption efficiencies (Figure 1.3) that limited the absorptive capacity, the effect was not strong enough as to counteract the benefits derived from ingesting more food, since higher absorption rates were measured systematically for fast growers (see Figure 1.4 and Table 1.4). Since no differences were observed in the energy expenditure between F and S mussels, growth differences recorded between S and F growing bivalves corresponded to enhanced feeding rates and higher digestive performance of F mussels, coupled to reduced metabolic costs per unit of assimilated food compared to S mussels.

These results are consistent with those previously reported for mussels, but also for oysters and clams (Toro et al. 1996; Toro and Vergara 1998; Bayne et al. 1999a, b; Pace et al. 2006; Tamayo et al. 2011, 2013, 2014, 2015; Fernández-Reiriz et al. 2016, Prieto et al. 2018 – chapter 2). In fact, broad differences in feeding rates between fast and slow growing individuals have been reported in so many occasions that it might be

concluded that a higher capacity for suspension feeding could be a general phenotypic trait of fast growing individuals. However, Tamayo et al. (2016) and Prieto et al. (2018) (chapter 2) found that whereas F mussels selected under optimal feeding conditions (continuous supply of high organic content diet) adjusted to this general approach, F mussels selected under nutritionally restricted conditions were capable of growing faster due to their capacity to save energy during the long periods of starvation. Actually, these reported differences found between the physiological profiles of mussels differentiated as F or S as a function of the environmental conditions led as to run these experiments. We aimed to find out whether or not forcing mussels to live for months under conditions where pseudofaeces production was imperative could also affect the physiological traits underlying the size differentiation by comparison with those known to operate below the pseudofeces production threshold. Contrary to our expectations, present results probed that the physiological basis of growth rate differences between F and S mussels were the same in mussels reared with diets below or above the pseudofeces threshold. Moreover, for all the physiological parameters measured, we found that the response of F mussels obtained from both rearing conditions were always very similar between them, suggesting that the innate differences that determine size differentiation when good nutritional conditions are provided, are quite the same. Indeed, as reported by Prieto et al. (2018), even under environmental conditions imposing moderate feeding restrictions (similar to the ones at tidal regimes) the same pattern was observed: fast feeding mussels grew faster, and only under severe (Tamayo et al. 2016) or very severe food restrictions (Prieto et al. 2018 – chapter 2) the differences between fast and slow growers turned to be based on the capacity to reduce metabolic expenditure. The fact that feeding conditions can affect the correlations between genetic (endogenous) factors and physiological rates is not new, it was earlier proposed by Bayne and Hawkins (1997) who based on correlations found between growth rates, multilocus heretozygosity, protein deposition efficiency and cost of growth, concluded that significant correlations between growth rate and heterozygosity were only found at high food rations. That is, genetic factors promoting differential protein turnover requirements became relevant under conditions of food abundance when energy incorporation dominated the energy balance, whereas lack of correlation at low rations suggested that the heterozygosity level did not modulate the lowest metabolic expenditure.

Our results also suggest that the endogenous differences underlying the differential physiological features that determine the differences in growing rate of F and S mussels could also differentially affect some morphological or morphofunctional characteristics, since we found that irrespective of the rearing condition, F mussels that showed higher clearance rates, also had significantly larger gill areas (see Figure 1.6). The high degree of plasticity of the gill was previously known (Theisen, 1982), and its relationship with the clearance rate has been addressed (Tendengren et al 1990), but until recently it was not analysed in relation to interindividual differences in growth rates. Larger gill areas in F individuals than in their S counterparts were reported for clams (Tamayo et al. 2011). But a striking result that may be pointed out is that, contrary to what it was found by Tendergren et al. (1990), in the present study, differences in the gill area were only related to its F or S nature (*growth condition*), and not to the nurture or *maintenance condition*, since F mussels reared under turbid or clear conditions had the same gill area, that was significantly larger that the area found for S_{AP} and S_{BP} mussels, whose gill area was very similar (see Table 1.5, ANOVA results). Thus, we concluded that endogenous factors regulating the expression of morphometric traits of this organ might play a major role in determining inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*. Differential gene expression in the gill tissues of F and S mussels was analysed by microarray techniques, and obtained results are presented in chapter 4, where the molecular basis determining differences in the CR was addressed.

The third aim of this study was to ascertain if the response to the variation of food quantity and quality in the environment was the same or not for F and S mussels reared at the tested maintenance conditions. The increase in the particle concentration of high-quality food (from H_L to H_H) promoted a general reduction of the clearance rate that led to the cancellation of most significant differences in SFG among groups. This same pattern of convergence between growth groups with the high food ration has been previously reported for mussels (Tamayo et al. 2016; Fernández-Reiriz et al. 2016), clams (Tamayo et al. 2011, 2013) and oysters (Tamayo et al. 2014). The CR reduction in response to increasing particle concentration of high organic content has been thoroughly reported in bivalves (since Foster-Smith, 1975) and interpreted as a mechanism allowing the regulation of ingestion rate and gut passage time (Thompson and Bayne, 1974; Bayne et al. 1987, 1988; Navarro et al. 1994). With low-quality diets,

increasing particle concentration (from L_L to L_H) promoted a less pronounced reduction of CR in all mussel groups. Such differences in CR behaviour of mussels fed diets of different qualities are consistent with previous findings in bivalves (Bayne et al. 1987; Navarro et al. 1994; Iglesias et al. 1996; Ward and Shumway, 2004). These differences have been explained by the capacity of bivalves to improve the absorption rate at high concentrations of low organic content food, by regulating the ingestion rate by means of rejecting filtered matter via pseudofaeces after a pre-ingestive selection of organically rich particles (Navarro and Iglesias, 1993; Urrutia et al. 1997). The higher ability of F mussels to select organic food items (as indicated by a significant effect of *growth condition* on SE) might have contributed to the higher SFGs attained by F individuals with the L_H diet.

It is widely recognized that bivalves have a considerable capacity to adjust feeding (i.e filtration, clearance, rejection and ingestion rates and selection efficiency) and digestive parameters (gut passage time, digestive enzyme activity, metabolic faecal losses) to cope with short- (hours, days) and medium-term (weeks) variations in the throphic conditions (Thompson and Bayne, 1974; Bayne et al. 1989, Navarro et al. 1991, 1994, 1996; Labarta et al. 1997, Ward and Shumway 2004, Bayne 2004, Albentosa et al. 2012). Regarding feeding, ingestion rate regulation by modifying CR and/or producing pseudofaeces at high concentration of low quality diets, coupled to a preferential ingestion of organic matter are the most remarkable adaptations. In our experiment, the variation of the physiological parameters among the experimental diets fitted the expected responses in physiological modification to variation on food characteristics, and clearance rates were found to exponentially increase with increasing concentration of organic matter (see Figure 1.1). Regarding this general pattern, we found a kind of differential response when comparing F and S mussels; changes in the feeding rate were in the same sense, but F mussels were able to modify their CR more markedly than S individuals (see the increase at low food concentrations as an example), and consequently they were able to better compensate or deal with food variations, so that their SFG kept less variable (see Figure 1.6). It could be that having higher gill surface areas allowed F individuals to enhance their capacity to increase CR when environmental trophic characteristics promoted the maintenance of high clearance rates; in fact, differences between F and S individuals decreased with rising food concentrations. More marked physiological differences between F and S individuals at

low food concentrations were also described by Tamayo et al. (2011 and 2016), who suggested, that fast growing individuals have a better homeostatic capacity (in terms of feeding). In this sense, Bayne (2004) considered some aspects of phenotypic plasticity in bivalves with an emphasis on feeding behavior and growth. He discussed the evidence for the existence of physiological trade-offs and described some of the proximate physiological processes that underlie individual growth differences, and the picture that emerged was one of considerable behavioral flexibility in response to changes in the food environment, linked with a strong genetic component to growth through a complex synergy of physiological traits. He concluded "there is no evidence to date that the faster rates of energy acquisition associated with increased growth rate act to constrain behavioral flexibility", and this is also what we found in this study.

Recalling the three hypothesis that were presented in the introduction, we could only accept the first one, since results supported that there were significant differences between F and S mussels reared under turbid conditions in the efficiency of the preingestive handling of particles, which contributed to a better growth performance of the fast growing mussels. However, this statement must be put into context, since fairly the same response was observed for mussels which were reared under low particle concentration conditions, which never before produced pseudofaeces until they were exposed to turbid conditions for just a week, revealing that a better capacity to cope with high seston loads was innate in F mussels, which apparently are endogenously provided to efficiently convert energy from the food into growth at almost any feeding condition, as long as there are no severe restrictions of food. The second and third hypothesis were based on the eventual differences in the physiological traits that were expected to result from the acclimation to the two different rearing conditions, but as explained before we could not find any difference between maintenance conditions, nor at the reference conditions, and neither when comparing the responses to several experimental conditions, so both those hypothesis were rejected.

In brief, having larger gills which allows displaying higher clearance rates and when necessary, to produce more pseudofaeces and select more efficiently organic matter for ingestion, coupled to a better digestive performance and reduced metabolic costs of growth are the key physiological features that are characteristic of the mussel phenotype that under good nutritional condition will develop as a fast grower. Present experiments have shown that, irrespective of the food concentration at which mussels

were reared, inter-individual growth potential differences resulting in size-differentiation are caused by the existence of endogenously determined differences in the feeding rate and the efficiency of the pre-ingestive processes for particle selection. Endogenous factors also regulate the expression of morphometric traits of the gill, and apparently play a major role in determining inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*. In order to clarify this aspect, differences in gene expression in gill tissues between F and S mussels have been analysed by microarray techniques, and results will be shown and discuss in chapter 4.

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Chapter 2

Mytilus galloprovincialis fast growing phenotypes under different restrictive feeding conditions: *fast feeders* and *energy savers*.

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Abstract

The present study aims to test if the environmental conditions prevailing during the growing period can determine the physiological profiles of specimens differentiated as fast (F) or slow (S) growers in the mussel *Mytilus galloprovincialis*. We reared mussel spats in the laboratory under two different conditions. In Treatment I (continuous feeding during discontinuous immersion), two mussel groups were submitted to a daily air exposure of 8 hs and fed continuously during immersion-time, with either high-quality food dosed below the pseudofaeces threshold (BP group) or low organic content food dosed above the pseudofaeces threshold (AP group). In Treatment II (discontinuous feeding during continuous immersion), mussels were continuously immersed but fed only 1 day per week (RC group). Mussels were reared for 7 and 11 months (time required for size-differentiation) in Treatments I and II, respectively, and the smallest and largest individuals from each group were selected as S and F specimens.

A series of feeding experiments (with different food quality, food ration and under continuous food supply) were performed to analyse the physiological performance of selected F and S mussels. In Treatment I, no significant differences were found in the metabolic rates between F and S mussels, and the faster growth rate

of F mussels resulted from their capacity to display higher clearance-ingestion rates and pre-ingestive selections. The physiological basis of growth rate differences between F and S mussels were found to be the same in mussels reared with diets below or above a pseudofaeces threshold (F_{BP}, F_{AP}, S_{BP} and S_{AP}). In contrast, the mussels from Treatment II had no significant differences in the feeding rates between F_{RC} and S_{RC} mussels. However, F individuals were found to have a 33% lower standard metabolic rate, indicating that fast growth under severe feeding restriction stemmed from a higher capacity of F mussels to save energy during long periods of starvation. Despite the differences in the physiological basis explaining fast growth between the two treatments, F mussels were found to possess significantly higher gill-surface area in both cases. It is thus concluded that endogenous factors affecting the gill-surface area play a major role in determining inter-individual growth rate differences in the mussel, *Mytilus galloprovincialis*.

Keywords: fast growing, ingestion rate, standard metabolic rate, scope for growth mussels

Introduction

Bivalve populations display high levels of endogenously determined inter-individual differences in growth rates. They have become good model systems for research on the physiological and metabolic basis for differential growth potential (Bayne 1999, 2000; Bayne et al. 1999a, 1999b; Toro et al. 2004; Tamayo et al. 2011, 2013, 2014, 2015, 2016), as well as for the analysis of the correlation between physiological functions and underlying genetic expression (Pace et al. 2006; Meyer and Manahan 2010; Wang et al. 2012; Bassim et al. 2014, 2015; Pan et al. 2015; De la Peña et al. 2016; Wilson et al. 2016). Studies of physiological energetics performed with different bivalve species collected from either natural populations or hatchery stocks have recognized the existence of endogenously caused inter-individual differences in the physiological components of the energy balance. Fast grower specimens have been reported to i) have significantly higher feeding rates (Bayne et al. 1999; Bayne 2000; Pace et al. 2006; Pernet et al. 2008; Tamayo et al. 2011, 2013, 2014, 2015, 2016); ii) express different types of digestive amylases (Prudence et al. 2006; Huvet et al. 2008); and iii) possess significantly higher gill-surface area (Tamayo et al. 2011). A higher

capacity for food processing has sometimes been found to combine in fast growers with lower metabolic costs associated with routine activities, such as food ingestion, absorption and tissue-growth (Bayne et al. 1999a, 1999b; Tamayo et al. 2013) or, alternatively, a lowered demand of energy to fulfil maintenance requirements (Bayne et al. 1999a; Pernet et al. 2008). The literature shows that the existence of inter-individual growth rate differences in bivalves may obey endogenous factors affecting the capacities for either acquiring, digesting and absorbing food or using metabolic energy in the processes of growth and maintenance.

We have recently shown (Tamayo et al. 2016) that environmental conditions, particularly food ambient, might play a crucial role in determining the physiological capacities of specimens differentiated as fast (F) versus slow (S) growers in the mussel *Mytilus galloprovincialis*. For instance, we observed that when mussel spats were reared under optimal trophic conditions, the fast growing individuals were found to possess higher mass-specific feeding rates, with same metabolic costs, than slow growers. However, when mussels were reared under restricted food conditions, fast growth resulted from a higher capacity of F mussels to save energy during starvation periods (lower mass-specific standard metabolic rates). These results suggest that, although many phenotypic features can potentially cause inter-individual growth rate differences, the environmental conditions, particularly nutritional conditions, act as a key factor determining the hierarchical order by which physiological traits contribute to promoting growth rate differentiation.

Natural populations of *Mytilus galloprovincialis* are submitted to alternate periods of food abundance and shortage due to seasonal cycles in phytoplankton growth, while also experiencing short-term tidal fluctuations in food availability and composition. The more obvious effect in the intertidal slope results from the aerial exposure, which restricts the time available for food consumption, while forcing the use of metabolic energy in developing compensatory responses against hypoxia, desiccation and temperature extreme variations (Widdows and Shick, 1985; Fitzgerald et al. 2012). Moreover, tide- and wind-driven water movements cause the resuspension of bottom sediments that promote the increment of particle concentration and the dilution of the available phytoplankton matter (Bayne 1993). The way in which individual bivalves are affected by fluctuations in particle concentration and quality depends on their ability to modulate food processing rates, either by rejecting lower quality particles or by

adjusting clearance rates (Winter 1976; Thompson and Bayne. 1974; Bayne et al. 1989, Hawkins et al, 1996, Navarro et al, 1994, 1996; Navarro and Widdows 1997).

In the present study, a series of experiments have been performed to analyse food restriction as a factor that can potentially affect the physiological processes underlying size differentiation. The laboratory experiments have been designed to mimic environmental conditions causing different types of food restriction events, such as reproducing tidal emersion regimes, long periods of food deprivation or sediment resuspension causing the dilution of available phytoplankton. For this purpose, juvenile mussels of *Mytilus galloprovincialis* of a homogeneous shell-length (approximately 10 mm) were collected from a natural rocky intertidal population and, in the laboratory, they were reared and left to size-differentiate (7 to 11 months) in two different treatments. Mussels in Treatment I (continuous feeding during discontinuous immersion) were submitted to a daily aerial exposure period of 8 hs and were fed continuously during the periods of immersion. In this treatment, mussels were divided into two groups, one group was fed a high-quality diet (high organic percentage) dosed at particle concentrations below the pseudofaeces threshold (BP diet), and the other group was fed a similar ration of phytoplankton but mixed with high concentrations of natural sediment, in order to provide a low-quality diet (low organic percentage) dosed at particle concentrations above the pseudofaeces threshold (AP). Mussels in Treatment II (discontinuous feeding during continuous immersion) were reared under continuous immersion regime, but submitted to a severe feeding restriction: they were fed a limited amount of phytoplankton only once per week. In each treatment, the specimens displaying the lowest and highest growth rates were selected, and the physiological components of their energy balance were determined in a series of feeding experiments with different combinations of food qualities and quantities. The aim of the study was to compare the physiological profiles of the mussels being selected as fast and slow growers in different conditions and try to confirm, or otherwise reject, the following working hypotheses:

1. Inter-individual differences in feeding rates (clearance rate) and metabolic costs of food processing and growth will contribute to the size differentiation (fast versus slow) of mussels reared under conditions of continuous feeding (Treatment I). No such differences are expected in mussels reared under conditions of severe feeding restriction (Treatment II).

2. Inter-individual differences in the standard metabolic rate will contribute to the size differentiation of mussels in both treatments. We expect that fast growers will have significantly lower SMR than slow growers.

3. Inter-individual differences in the ability to reject low-quality particles will contribute to the size differentiation of mussels that were reared with a diet dosed above the pseudofaeces threshold (AP diet). No such differences are expected in mussels reared with a diet dosed below the pseudofaeces threshold (BP diet).

4. Significant differences in the clearance rate between F and S mussels would be associated with significant inter-individual differences in the gill area.

Material and methods

Experimental design

Juvenile specimens of the mussel *Mytilus galloprovincialis* were collected in a rocky shore from Antzoras (Bizcay, North Spain, 43°24'29.1"N; 2°40'51.0"W) in February 2014. Approximately 520 individuals of 10 mm shell length and 150 mg live weight were separated into two groups and maintained in the laboratory during a long period (7 to 11 months) under two different sets of conditions (Treatment I and Treatment II).

Treatment I**:** Continuous feeding during discontinuous immersion. Two groups of 200 mussels were maintained in a simulated tidal regime consisting of 16 hs immersion in seawater and 8 hs of air exposure at room temperature (≈22 ºC). During the immersion period, mussels were fed with mixtures of the algae *Isochrysis galbana* (T-iso), lyophilized *Phaeodactilum tricornutum* and freshly collected and sieved particles of natural sediment. One group was maintained with a diet of high phytoplankton content, which was designed to have approximately 80% of organic matter, and was dosed at a total particulate matter concentration (TPM) of approximately 1.0 mg/L (BP diet: Below the pseudofaeces threshold). The second group was fed a similar ration of phytoplankton that was "diluted" in its organic content by the addition of natural sediment to provide a diet of low organic content (≈30%) that was dosed at TPM concentration (≈ 4.5 mg/L) above the pseudofaeces threshold (AP diet). During the period of immersion, the tanks were cleaned, and mussels were pulled apart

one from each other to avoid inter-individual competition for food during the next immersion period. During immersion, seawater was continuously aerated, the temperature was regulated at a constant value (16ºC) and the corresponding diet was continuously added to the tanks with peristaltic pumps from concentrated stocks. The shell-length and live-weight of specimens was monitored once per two weeks using 0.05 mm accuracy callipers and an 0.01 mg accuracy balance. Mussels were maintained under these conditions until clear size differences among specimens were found (7 months). After this period, the largest and smallest 24 individuals, representing the percentiles $P_{12.5}$ and $P_{87.5}$ in size distribution of each group, were selected as fast (F) and slow (S) growers, respectively. Accordingly, four experimental groups of mussels were obtained combining maintenance (BP and AP) and growth (F or S) conditions:

1. Fast growers selected below the pseudofaeces threshold (F_{BP});
2. Slow growers selected below the pseudofaeces threshold (S_{BP});
3. Fast growers selected above the pseudofaeces threshold (F_{AP}); and
4. Slow growers selected above the pseudofaeces threshold (S_{AP}).

To analyse the differences in the physiological performance of F_{BP}, S_{BP}, F_{AP} and S_{AP} mussels, 6 individuals from each of the 4 groups were used in feeding experiments, and the physiological components of their energy balance were determined. The feeding experiments included 4 experimental diets resulting from the combination of 2 food qualities and 2 food concentrations: i) high-quality, low concentration (H_L); ii) high-quality, high concentration (H_H); iii) low-quality, low concentration (L_L); and iv) low-quality, High concentration (L_H).

Treatment II: Discontinuous feeding during continuous immersion. Approximately 120 individuals were confined in aerated seawater tanks at a constant temperature (16 ºC) and submitted to a discontinuous and severely restricted food regime. Mussels were fed only once per week by abruptly adding a known volume (3 L) of phytoplankton (*Isochrysis galbana*) culture to the tank. The addition of phytoplankton promoted a sudden increase in particle concentration that was gradually reduced due to the filtering action of the mussels. No more food was added, and the mussels were starved for the remainder of the week (6 days). After 11 months, the 12 largest and smallest mussels, representing the percentiles P_{20} and P_{80} in size distribution, were selected as F and S mussels (F_{RC} and S_{RC}) to analyse the differences in the

physiological performance between F_{RC} and S_{RC} mussels. Samples of 6 individuals were used in feeding experiments. The feeding experiments with selected F_{RC} and S_{RC} mussels were organized as follows. Mussels were fed the usual phytoplankton dose and starved for 6 days; afterwards, the standard metabolic rate (SMR) of each individual was measured. Once the SMR was determined, mussels were divided into two groups and fed one of the two diets included in the experimental design: H_L and H_H diets. The diets were made of phytoplankton (*Isochrysis galbana*) and a small proportion of silt particles to provide for 80% organic percentage in the diet, and they were dosed at low (H_L) or high (H_H) particle concentration. The mussels were submitted to a continuous food supply regime from 11 to 12 days. During that period, the clearance rate of each individual was monitored. The complete set of physiological parameters underlying energy balance were determined in two occasions: after 1 day of exposure to the diet (acute response) and after 11 (with H_L diet) or 12 (with H_H diet) days of feeding (acclimated response).

Experiments of physiological energetics with selected fast and slow growing mussels

Characteristics of diets

Diets for feeding experiments with selected F and S mussels consisted of mixtures of cells of *Isochrysis galbana* and particles of freshly collected natural sediment. Samples of food suspensions were filtered onto ashed, pre-weighed GF/C glass-fiber filters and subsequently processed to determine total particle matter concentration (TPM: mg/L), inorganic particulate matter (PIM: mg/L) and organic particulate matter (POM: mg/L). Retained salts were rinsed out with a solution of ammonium formate (0.9%). TPM and PIM were estimated as the dry and ash weight increment of the filters, respectively. POM was calculated as the difference between TPM and PIM. Organic content (f) was estimated as POM/TPM.

Physiological measurements

Energy acquisition

Clearance rate (CR: L/h) was measured according to Hildreth and Crisp (1976) as:

$$CR = F^* ((C_i - C_o)/C_i)$$

where F is the flow rate (L/h), and C_i and C_o represent the particle concentration in the inflow and outflow of the chamber, respectively. Particle concentration was determined with a Coulter Counter Z1. Specimens were placed individually in the chambers. Seawater flow rates through the chambers were adjusted to obtain a particle concentration reduction of 15-30%. Individual measurements of CR were taken every hour during a period of 11-12 hours. *Filtration rates* of total (FR: mg/h) and organic (OFR: mg/h) matter were then calculated as CR*TPM and CR*POM, respectively. In the absence of pseudofaeces production, filtration rates of total and organic matter were considered to represent the *ingestion rate* of total (IR: mg/h) and organic matter (OIR: mg/h), respectively. *Absorption efficiency* (AE: Decimal units) was determined according to the Conover method (Conover, 1966) as:

AE= (f-e)/(1-f)*e

where f and e represent the organic content of food and faeces, respectively.

Finally, *Absorption rate* (AR: mg/h) was then calculated as OIR *AE.

Mussels produced pseudofaeces with the L_H diet in Treatment I. An exhaustive collection of the pseudofaeces produced by each individual mussel during the period of CR determination allowed the computation of *rejection rates* of total (RR: mg/h) and organic matter (ORR: mg/h). The ingestion rates of total and organic matter were then computed as the differences between the filtration and rejection of total (IR= FR-RR) and organic matter (OIR= OFR-ORR), respectively. An exhaustive collection of the faeces produced by each individual mussel during the period of CR determination allowed the computation of the *egestion rate* of total (ER: mg/h) and organic matter (OER: mg/h). Thus, the absorption rate (AR) was calculated as OIR-OER and absorption efficiency was calculated as AE/OIR. The efficiency of the pre-ingestive selection process (SE: fraction) was estimated as SE = 1-(p/f), where p and f represent the organic content of the pseudofaeces and food, respectively.

Metabolic expenditure

After the determination of food acquisition rates, mussels were introduced in individual chambers (150 mL) sealed with LDO oxygen probes connected to oxymeters (HATCH HQ 40d) for the determination of routine oxygen consumption (VO_{2R}: mL O_2/h). The rates of VO_2 were derived from the decrease of the oxygen concentration of the water over time. Water oxygen concentration data were registered every 5-10

minutes until oxygen values decreased by 20-30% of the initial baseline. A control chamber was used to check the stability of the oxygen concentration. Subsequently, in Treatment I, mussels were starved for seven days, and the rates of oxygen consumption were measured again to determine the standard oxygen consumption (VO_{2S}: mL O_2/h). In Treatment II, VO_{2S} was measured before the feeding experiments.

The routine metabolic rate (RMR: J/h) and standard metabolic rate (SMR: J/h) were estimated from routine and standard oxygen consumption using an oxycaloric coefficient of 20.08 J/mL O_2 (Gnaiger, 1983).

Energy balance

Scope for growth (SFG: J/h) was estimated as the difference between absorbed energy (AR: J/h) and metabolic expenditure (RMR: J/h). AR (mg/h) was transformed into energetic values (J/h) using an energy equivalence of 18.75 J/mg (Whyte, 1987).

Size standardization of the physiological rates.

All physiological rates were standardized to an equivalent 50 mg dry mass mussel, according to the following expression (Bayne and Newell, 1983):

$$Y_{STD} = (50/M_{EXP})^b * Y_{EXP},$$

where Y_{STD} and Y_{EXP} represent the standard and experimental physiological rates, respectively, and M_{EXP} stands for the experimental mass of the mussel. The power value that scales physiological rates to body weight (b) used for clearance rate and oxygen consumption were 0.58 (Bayne and Hawkins, 1997) and 0.724 (Bayne et al. 1973), respectively.

Gill-surface area (GA:mm^2)

After the determination of physiological measurements, mussels were dissected and placed on graph paper for setting the scale. A photograph of the internal tissues of each mussel was taken with a digital camera, and the gill-surface area of each individual mussel was then calculated using *ImageJ* program. The displayed data correspond to one side of a demibranch. Gill areas were standardized for an equivalent 20 mm shell-length mussel according to the expression:

$$GA_{STD} = (20/L_{EXP})^b * GA_{EXP},$$

where GA_{STD} and GA_{EXP} represent the standardized and experimental gill area, respectively, and L_{EXP} is the experimental shell-length of the mussel. The power function that scales gill area to shell-length was 1.85 (Perez Camacho and Gonzalez, 1984).

Statistical analysis

Average growth rate of mussels reared with diets below and above pseudofaeces threshold (BP and AP) in Treatment I was estimated by fitting mean values of shell-length (y) and time (x) to linear regression models (least square process). Significant differences in growth rates between mussels grown with BP and AP diets was tested by comparing the slope (b) and intercepts (a) using covariance (ANCOVA) procedures described in Zar (2010). With selected mussels from treatment I, the effect of *growth condition* (F versus S) and *maintenance condition* (BP versus AP) on the physiological parameters of mussels fed each experimental diet was tested using a two-way factor ANOVA. Significant effects of *growth condition* and *maintenance condition* in the gill-surface areas of mussels were tested using a two-way factor ANOVA. Normality and homogeneity of variances were tested using Shapiro-Wilk and Levene´s test, respectively, prior to the analysis of the data. Differences between mussel-groups were analysed by post hoc tests, Games Howell or Tukey, according to the Levene´s test results. The relationship between routine metabolic rate and absorption rate of F and S mussels from Treatment I was fitted to linear regressions following least square process and significant differences in slopes (b) and intercepts (a) of between them was tested by performing covariance (ANCOVA) analysis.

With mussels from treatment II, the significant variations in the clearance rate of F and S mussels along the period of acclimation to continuous feeding was analyzed by performing repeated measurements ANOVA, using LSD post-hoc tests. Data sphericity was tested by Mauchly´s test. Accordinly, univariate statistical approach (Asumed sphericity test) or multivariate approaches (Pilai´s trace test) was used. Significant differences between F and S in daily CR were tested using student´s t-test. Significant differences in the physiological components of the energy balance between F and S individuals in the acute (day 1) and acclimated (day 11 or 12) in mussels from Treatment II were tested using Student´s t-test. The effect of *exposure time* (acute versus acclimated) on the physiological parameters of these mussels was tested using

paired t-test. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp. Released 2010. Armonk, NY: IBM Corp.)

Results

Treatment I: Continuous feeding during discontinuous immersion

Growth rate and selection of fast and slow growers

The growth rates (GR: mm/day) of mussels grown under BP and AP diets were estimated by adjusting mean values of shell-length to linear regression models. The resulting equations are:

BP: GR = 0.0364(±0.001) *time + 11.645 (±0.060), t = 195.58, p<0.0001

AP: GR = 0.0367(±0.001) *time + 12.525 (±0.058), t = 217.19, p<0.0001

Mussels grew approximately 0.037 mm/day with both food qualities. The ANCOVA indicates a lack of significant differences in growth rate (Slope test: t=0.203, df=1, 8, p>0.05; "intercept" test: t=-12.56, df=1, 8, p>0.05). After 190 days of maintenance, the largest and the smallest 24 individuals were selected in both feeding tanks and considered to represent F and S growers¸ respectively. Fast growing mussels had 61% larger shell-length and 3-fold higher live-weight than their slow growing counterparts in both diets (Table 2.1).

Table 2.1. Shell-length (mm) and live weight (g) (mean ± SD) of mussels of Treatment I. Initial values upon arrival to the laboratory (n= 200) and values corresponding to mussels selected as fast and slow growers (n=24) after 190 days of maintenance.

	Initial	F_{BP}	S_{BP}	Initial	F_{AP}	S_{AP}
Length (mm)	11.8 ± 0.30	22.9 ± 1.19	14.2 ± 0.69	12.9 ± 0.46	24.3 ± 1.18	15.1 ± 0.77
Weight (g)	0.3 ± 0.03	1.4 ± 0.21	0.4 ± 0.05	0.3 ± 0.05	1.5 ± 0.19	0.5 ± 0.66

Experiments of physiological energetics with selected F and S mussels

Characteristics of experimental diets (H_L, H_H, L_L and L_H) used in feeding experiments are shown in Table 2.2.

Table 2.2. Characteristics of the experimental diets used in feeding experiments with mussels from Treatment I. TPM: total particulate matter; PIM: particulate inorganic matter; POM: particulate organic matter and f: organic content. H_L: high-quality low concentration; H_H: high-quality high concentration, L_L: low-quality low concentration, L_H: low-quality high concentration.

Diet	TPM (mg/L)	PIM (mg/L)	POM (mg/L)	f (fraction)
H_L	0.43 ± 0.02	0.09 ± 0.02	0.35 ± 0.03	0.80 ± 0.05
H_H	1.71 ± 0.17	0.61 ± 0.08	1.10 ± 0.12	0.64 ± 0.04
L_L	0.88 ± 0.08	0.54 ± 0.05	0.34 ± 0.03	0.38 ± 0.02
L_H	3.87 ± 0.29	2.98 ± 0.26	0.89 ± 0.02	0.23 ± 0.01

Physiological measurements of energy balance

The clearance rates of mussels from the 4 groups (F_{BP}, S_{BP}, F_{AP} and S_{AP}) in the four experimental diets (H_L, H_H, L_L and L_H) are shown in Figure 2.1. Significant effects of *maintenance condition* (BP vs. AP mussels) and *growth condition* (F vs. S mussels) on CR of the mussels were tested by performing a two-factor analysis of variance, and the results of these analyses are summarized in Figure 2.1. With high-quality diets (Figure 2.1a), two trends of CR were evident: 1) at low food concentrations (H_L diet), fast growing mussels (both F_{BP} and F_{AP}) displayed higher CRs (approximately two-fold higher, significant in AP mussels) than slow growing mussels (S_{BP} and S_{AP}); thus, the *growth condition* was found to exert a significant effect ($p<0.001$) in the two-factor ANOVA. 2) With the high food ration (H_H), CR was found to be lower in all mussel groups compared with the H_L diet. Such a reduction was higher in F (both F_{BP} and F_{AP}) mussels than in S mussels. In the H_L diet, a significant effect of *maintenance condition* was recorded in the ANOVA ($p= 0.011$). The post hoc test indicates that F_{AP} mussels had significantly higher CRs than mussels from BP treatment (F_{BP} and S_{BP}).

A similar pattern of CR was observed with mussels feeding on low-quality diets (Figure 2.1b): 1). With the low ration (L_L), the *growth condition* was found to exert a highly significant effect (p value<0.001), accounting for the fact that fast growing mussels (both F_{BP} and F_{AP}) attained significantly higher CRs than slow growers (S_{BP} and S_{AP}). With the high food ration (L_H), the overall CR of F mussels was reduced compared with the L_L diet. No significant differences in CR were found between mussel groups fed the H_H diet.

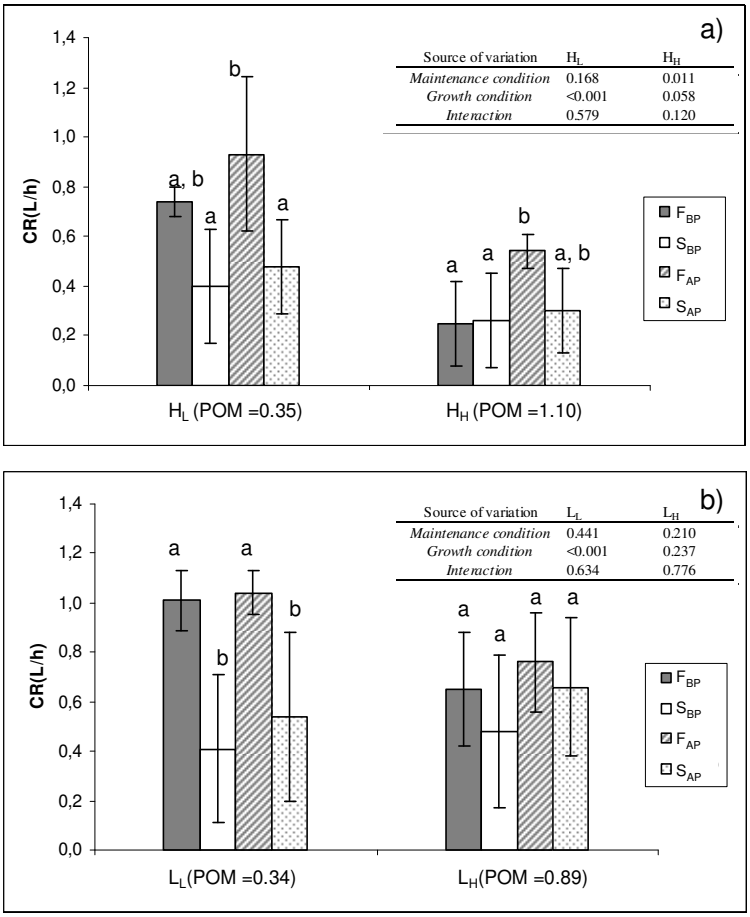

Figure 2.1. Clearance rate (L/h) of F_{BP}, S_{BP}, F_{AP} and S_{AP} mussels from Treatment I fed experimental high-quality diets (a) and low-quality diets (b). Letters indicate significant differences between F and S mussels. At the upper side, the p values of two-way factor ANOVAs testing significant effects of growth condition (F or S) and maintenance condition (BP or AP) are shown.

The remaining components of the energy balance are shown in Table 2.3, and the summaries of the two-way ANOVA analyses testing significant effects of *growth condition* (F vs. S) and *maintenance condition* upon physiological parameters are shown in Table 2.4. The resulting scope for growth in mussels from the 4 groups (F_{BP}, S_{BP}, F_{AP} and S_{AP}) in the four experimental diets (H_L, H_H, L_L and L_H) and the summary of the two-way ANOVA has been plotted in Figure 2.2. The entire two-way ANOVA table for every analysis done for all the physiological parameters has been included as electronic supplementary material (Table 2.A1)

H_L diet. In agreement with CR, *growth condition* exerted a significant effect on the ingestion of organic matter (Table 2.3 and ANOVA in Table 2.4). Since no significant differences were recorded in the absorption efficiency between mussels (AE ranged 0.60-0.70), the absorption rate followed the similar trend to that of OIR, where F_{BP} and F_{AP} mussels showed higher ARs than S_{BP} and S_{AP} mussels, attaining statistical significance in mussels from BP. The routine oxygen consumption was found to be rather constant between mussel groups (≈0.45 mL O_2/h) and, thus, inter-group differences in SFG followed the same trend to that exhibited by CR and AR (Figure 2.2a). The post hoc analyses indicate that differences in SFG between F and S mussels attained the required statistical significance only with BP mussels (F_{BP} vs. S_{BP}); with AP mussels, differences were close to a statistical significance (p value = 0.069). The standard metabolic rate was significantly affected by *growth condition* (p value=0.018, Table 2.4): fast growers tend to have higher VO_{2S} than slow growers.

H_H diet. The absorption efficiency ranged from 0.40 to 0.54 and was not significantly different between mussels. Therefore, inter-group differences in AR followed the same pattern to that of CR and OIR. F_{AP} mussels displayed significantly higher AR than F_{BP} and S_{BP} mussels, and there was a significant effect of the *maintenance condition* factor. The VO_{2R} was higher in AP mussels than in BP mussels; this accounted for a significant effect of *maintenance condition* in the two-way factor ANOVA (p value= 0.033). Despite their higher VO_{2R} values, due to their high absorption rates, F_{AP} mussels also attained the highest SFG (Figure 2.2a). No significant effects were found for the VO_{2S}.

L_L diet. In agreement with CR values (Figure 2.1b), fast growing mussels (F_{BP} and F_{AP}) displayed significantly higher (approximately double) OIR than slow growing mussels (S_{BP} and S_{AP}). Furthermore, AE (ranged 0.51 to 0.69) was also significantly higher in F mussels and, consequently, the resulting AR was more than two-fold higher in fast than in slow growers. Thus, significant effects of *growth condition* on OIR, AE and AR were recorded in the ANOVA (Table 2.4). The routine oxygen consumption followed the same pattern of AR and was significantly higher (p value= <0.001, Table 2.3) in fast growers (67% higher in BP mussels and 40% higher in AP mussels). The resulting SFG was two- to three-fold higher in fast growers (Figure 2.2b). No significant effects were observed for the VO_{2S}.

Table 2.3. Physiological parameters (mean ± SD) measured in mussels during feeding experiments of Treatment I. Diets: i) high-quality low concentration (H_L), ii) high-quality high concentration (H_H), iii) low-quality low concentration(L_L) and iv) low-quality high concentration (L_H). Mussel groups: i) fast grower below pseudofaeces threshold (F_{BP}), ii) slow grower below pseudofaeces threshold (S_{BP}), iii) fast grower above pseudofaeces threshold (F_{AP}), iv) slow grower above pseudofaeces threshold (S_{AP}). Physiological parameters: CR: clearance rate (L/h), SE: selection efficiency (fraction), RP: rejection proportion OIR: organic ingestion rate (mg/h), AE: absorption efficiency (fraction), AR: absorption rate (mg/h), VO_{2R}: routine oxygen consumption (mL/h), VO_{2S}: standard oxygen consumption (mL/h) and SFG: scope for growth (J/h). Letters indicate statistical aggrupation of growth groups per parameter according to the corresponding post hoc test.

	F_{BP}	S_{BP}	F_{AP}	S_{AP}
H_L	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
OIR (mg/h)	0.26 ± 0.02 $^{a.b}$	0.14 ± 0.08 ^{a}	0.32 ± 0.11 ^{b}	0.17 ± 0.07 ^{a}
AE (fraction)	0.65 ± 0.12^{a}	0.60 ± 0.07^{a}	0.62 ± 0.13^{a}	0.70 ± 0.16^{a}
AR (mg/h)	0.17 ± 0.04^{a}	0.08 ± 0.04^{b}	0.19 ± 0.06^{a}	0.12 ± 0.05$^{a.b}$
VO_{2R} (mL/h)	0.045 ± 0.005^{a}	0.046 ± 0.017^{a}	0.046 ± 0.017^{a}	0.041 ± 0.019^{a}
VO_{2S} (mL/h)	0.026 ± 0.006 ^{a}	0.021 ± 0.013 ^{a}	0.027 ± 0.008 ^{a}	0.013 ± 0.007 ^{a}
H_H				
OIR (mg/h)	0.28 ± 0.19 ^{a}	0.28 ± 0.20 ^{a}	0.59 ± 0.07 ^{b}	0.33 ± 0.19 $^{a.b}$
AE (fraction)	0.41 ± 0.12 ^{a}	0.54 ± 0.12 ^{a}	0.49 ± 0.05 ^{a}	0.49 ± 0.05 ^{a}
AR (mg/h)	0.14 ± 0.12 $^{a.b}$	0.13 ± 0.07 ^{a}	0.28 ± 0.03 ^{b}	0.16 ± 0.10 $^{a.b}$
VO_{2R} (mL/h)	0.054 ± 0.018^{a}	0.046 ± 0.018 ^{a}	0.066 ± 0.012 ^{a}	0.062 ± 0.003 ^{a}
VO_{2S} (mL/h)	0.035 ± 0.014 ^{a}	0.043 ± 0.022 ^{a}	0.050 ± 0.013 ^{a}	0.043 ± 0.019 ^{a}
L_L				
OIR (mg/h)	0.34 ± 0.04 ^{a}	0.14 ± 0.10 ^{b}	0.35 ± 0.03 ^{a}	0.18 ± 0.11 ^{b}
AE (fraction)	0.69 ± 0.02 ^{a}	0.51 ± 0.14 ^{a}	0.67 ± 0.052 ^{a}	0.56 ± 0.19 ^{a}
AR (mg/h)	0.24 ± 0.03 ^{a}	0.09 ± 0.07 ^{b}	0.24 ± 0.02 ^{a}	0.12 ± 0.08 $^{a.b}$
VO_{2R} (mL/h)	0.057 ± 0.004 ^{a}	0.034 ± 0.011 ^{b}	0.049 ± 0.012 $^{a.l}$	0.035 ± 0.017 ^{b}
VO_{2S} (mL/h)	0.033 ± 0.01 ^{a}	0.022 ± 0.009 ^{a}	0.020 ± 0.005 ^{a}	0.027 ± 0.014 ^{a}
L_H				
SE (fraction)	0.51 ± 0.05 ^{a}	0.44 ± 0.05 ^{a}	0.54 ± 0.02 ^{a}	0.44 ± 0.07 ^{a}
RP (fraction)	0.52 ± 0.12 ^{a}	0.67 ± 0.05 ^{a}	0.49 ± 0.07 ^{a}	0.50 ± 0.09 ^{a}
OIR (mg/h)	0.43 ± 0.15 ^{a}	0.28 ± 0.17 ^{a}	0.52 ± 0.13 ^{a}	0.43 ± 0.18 ^{a}
AE (fraction)	0.71 ± 0.07 ^{a}	0.68 ± 0.04 ^{a}	0.68 ± 0.03 ^{a}	0.62 ± 0.05 ^{a}
AR (mg/h)	0.31 ± 0.11 ^{a}	0.23 ± 0.09 ^{a}	0.36 ± 0.10 ^{a}	0.30 ± 0.07 ^{a}
VO_{2R} (mL/h)	0.075 ± 0.012^{a}	0.069 ± 0.024 ^{a}	0.071 ± 0.013 ^{a}	0.067 ± 0.022 ^{a}
VO_{2S} (mL/h)	0.045 ± 0.012 ^{a}	0.045 ± 0.007 ^{a}	0.047 ± 0.013 ^{a}	0.042 ± 0.015 ^{a}

Table 2.4. P values of two-way factor ANOVAs testing significant effects of *growth condition* (F or S) and *maintenance condition* (BP or AP) on physiological parameters of mussels when fed the four experimental diets (H_L, H_H, L_L and L_H) of Treatment I.

	H_L	H_H	L_L	L_H
SE				
Maintenance condition				0.634
Growth condition	n.d.	n.d.	n.d.	**0.011**
Interaction				0.921
RP				
Maintenance condition				**0.034**
Growth condition	n.d.	n.d.	n.d.	0.074
Interaction				0.139
OIR				
Maintenance condition	0.168	**0.012**	0.440	0.103
Growth condition	**<0.001**	0.059	**<0.001**	0.099
Interaction	0.583	0.120	0.634	0.719
AE				
Maintenance condition	0.497	0.737	0.656	0.054
Growth condition	0.690	0.089	**0.006**	0.098
Interaction	0.230	0.120	0.663	0.461
AR				
Maintenance condition	0.138	**0.020**	0.392	0.179
Growth condition	**<0.001**	0.057	**<0.001**	0.171
Interaction	0.794	0.207	0.578	0.802
VO_{2R}				
Maintenance condition	0.762	**0.033**	0.559	0.651
Growth condition	0.740	0.307	**0.001**	0.566
Interaction	0.596	0.856	0.356	0.866
VO_{2S}				
Maintenance condition	0.355	0.309	0.398	0.893
Growth condition	**0.018**	0.980	0.683	0.705
Interaction	0.240	0.332	0.061	0.648

L_H diet: High food concentration and low organic matter proportion promoted the production of pseudofaeces in mussels from the four experimental groups. Mussels rejected 49% to 67% of the filtered matter (Table 2.3), and the two-factor ANOVA indicated a significant effect of *maintenance condition* (p=0.034, Table 2.4), accounting for the fact that mussels reared above the pseudofaeces threshold (AP mussels) reject a lower proportion of matter than those reared below the pseudofaeces threshold (BP mussels). The mean selection efficiency ranged from 51-54% in fast growing mussels but was lower (44%) in slow growing mussels; thus, *growth condition* appears as a significant factor (p value= 0.011) in the two-way ANOVA (Table 2.4). No significant effects of *maintenance condition* or *growth condition* were recorded for CR, OIR, AE, AR, VO_{2R} and VO_{2S}. In regards to SFG, fast growing mussels were approximately two-

fold higher than in slow growing individuals (Figure 2.2b). Accordingly, a significant effect of *growth condition* on SFG is recorded in the two-way ANOVA (p value= 0.031).

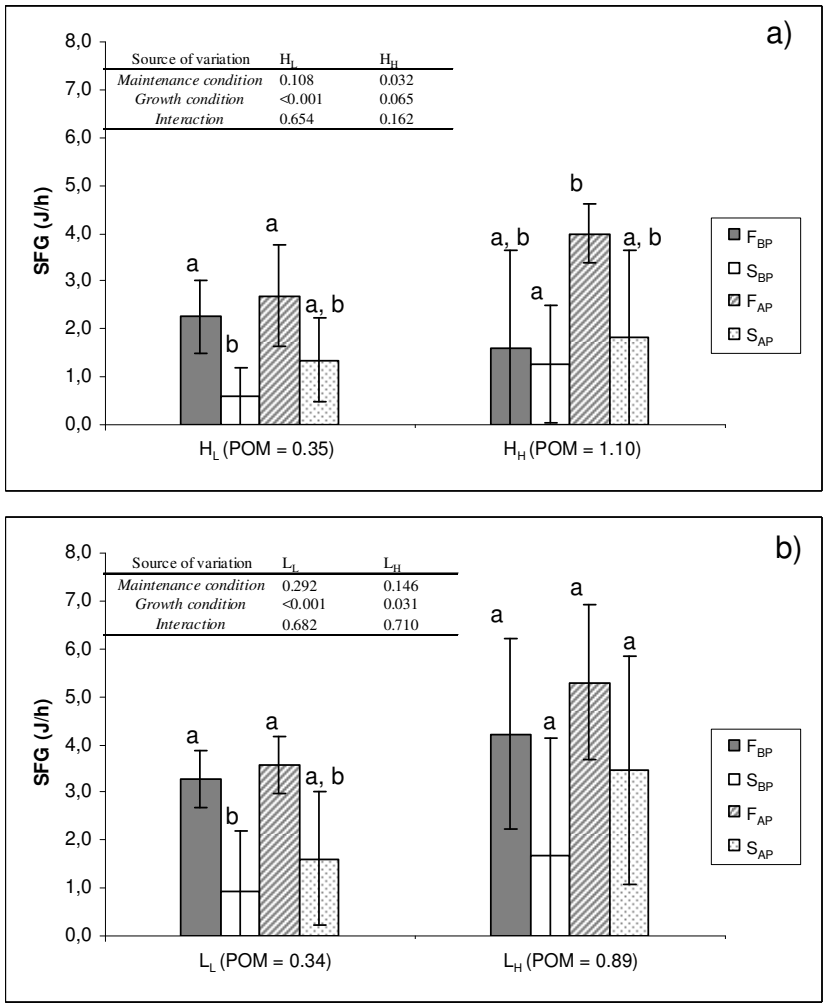

Figure 2.2. Scope for growth (J/h) of F_{BP}, S_{BP}, F_{AP} and S_{AP} mussels from Treatment I fed experimental high-quality diets (a) and low-quality diets (b). Letters indicate significant differences between F and S mussels. At the upper side, the p values of two-way factor ANOVAs testing significant effects of growth condition (F or S) and maintenance condition (BP or AP) are shown.

The metabolic cost of feeding, digestion and absorption (MSFG) were estimated from regression lines fitting data points for the routine metabolic rate (J h^{-1}) vs. absorption rate (J h^{-1}), and lines were compared for F and S mussels from different groups in Treatment I in order to analyse possible differences between growth groups. The corresponding regression lines were:

F mussels: RMR = 0.129(±0.020) *AR + 0557 (±0.098), F=42.178, p<0.0001 n=44

S mussels: RMR = 0.138(±0.029) *AR + 0.630 (±0.092), F=23.882, p<0.0001 n=41

No significant differences between slopes and elevations were found between F and S mussels in the ANCOVA analysis (Slope test: t=0.263, df=1, 81, p>0.05; "intercept" test: t=1.46, df=1, 81, p>0.05). Therefore, a single regression equation relating RMR and AR has been plotted in the Figure 2.3 (Slope=0.133; a=0.587).

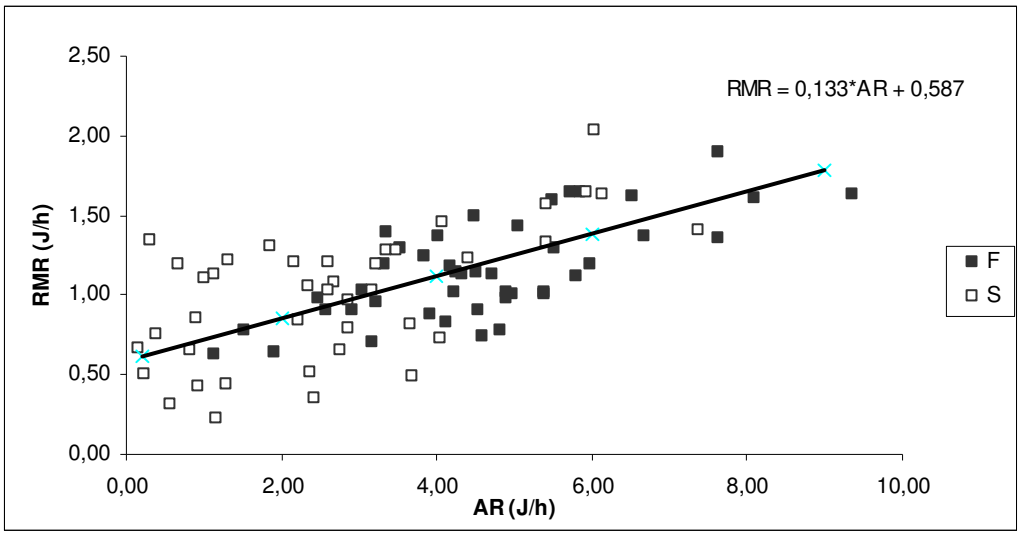

Figure 2.3. Routine metabolic rate (RMR: J/h) as a function of absorption rate (AR: J/h) on F (full squares) and S (empty squares) mussels from Treatment I.

Gill-surface area of fast and slow growing mussels

Mean values (± SD) of the gill area are shown in Figure 2.4. The two-factor ANOVA performed to test the significant effect of *growth condition* and *maintenance condition* on the gill area of the mussels is shown in the figure. *Growth condition* and *maintenance condition* exerted significant effects on the gill area: F mussels and mussels from the AP maintenance condition had higher gill areas. A non-significant effect of the interaction term *growth condition * maintenance condition* was found.

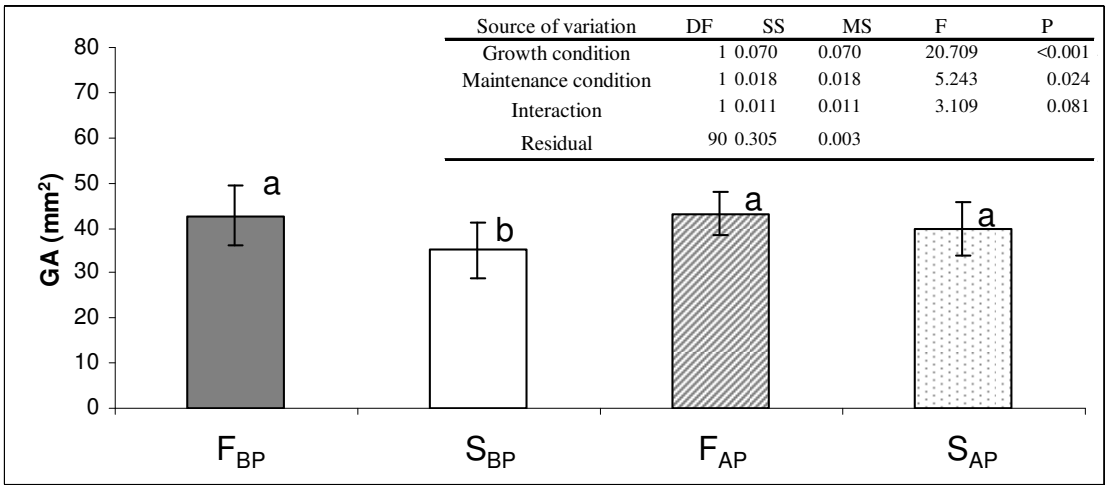

Figure 2.4. Gill surface-area (GA: mm^2) of F_{BP}, S_{BP}, F_{AP} and S_{AP} mussels from Treatment I. Letters indicate significant differences between groups (post hoc Tukey test). At the upper side, the two-way factor ANOVA testing significant effects of growth condition (F or S) and maintenance condition (BP or AP) is shown.

Treatment II: Discontinuous feeding during continuous immersion

Growth rate and selection of fast and slow growers

The shell-length and live-weight of fast (F_{RC}) and slow (S_{RC}) growing mussels after the maintenance period on Treatment II are shown in Table 2.5. Shell-length and live-weight were 32% and 66% higher, respectively, in F_{RC} compared with S_{RC} mussels.

Table 2.5. Shell length (mm) and live weight (g) of fast (F) and slow (S) growing mussels selected in Treatment II.

	F_{RC}	S_{RC}
Length (mm)	21.1 ± 0.73	15.9 ± 0.81
Weight (g)	1.0 ± 0.12	0.6 ± 0.01

Feeding experiments with selected F_{RC} and S_{RC} mussels

Characteristics of the diets: The two experimental diets had a common organic content of approximately 85%, and the total particle matter concentrations (TPM: mg/L) were 0.55 and 2.0 in H_L and H_H, respectively.

The *standard oxygen consumption* was significantly lower (approximately 33% lower) in F_{RC} mussels (Figure 2.5) than in S_{RC} mussels.

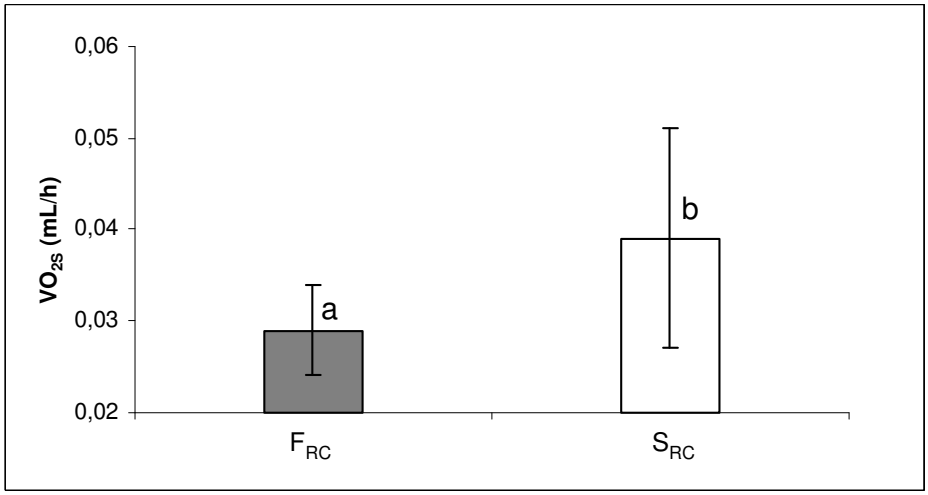

Figure 2.5. Standard oxygen consumption (VO_{2S}: mL O_2/h) of F_{RC} (solid bar) and S_{RC} (empty bar) mussels from Treatment II. Letters indicate significant differences between groups tested by Student´s t-test (p value= 0.01)

Physiological measurements of energy balance

H_L diet. On the first day of feeding the H_L diet, no differences in CR were found between F_{RC} (0.54±0.09 L/h) and S_{RC} (0.51±0.22 L/h) mussels (Figure 2.6a). However, CR diverged progressively and significant differences were found and the days 6, 8 and 11. The remaining physiological components of the energy balance in mussels fed the H_L diet were recorded only on the first day (acute response) and the last day of the experiment (acclimated response). The results are shown in Table 2.6. For the acute response, no significant differences were found in the main physiological parameters (CR, AR, VO_{2R} or SFG) between F and S mussels; only AE was found to be slightly higher in F than in S mussels. During the experimental period of feeding, AE increased 12% and 22% in F_{RC} and S_{RC} mussels, respectively, and VO_{2R} increased significantly as much as 46% in both groups of mussels (Table 2.6). After acclimation (11 days), F_{RC} mussels displayed significantly higher CRs than S_{RC} mussels (≈ two-fold higher: 0.63 ± 0.20 vs. 0.32 ± 0.23 L/h in F_{RC} and S_{RC} mussels, respectively, Figure 2.6a). In good correspondence with higher CRs, SFG was 4 times higher in F_{RC} mussels at the end of the experiment (1.63 ± 0.58 and 0.42 ±1.46 in F_{RC} and S_{RC}, respectively). However, the

standard deviation in S_{RC} mussels was high, and differences did not attain the required statistical significance (p value=0.122).

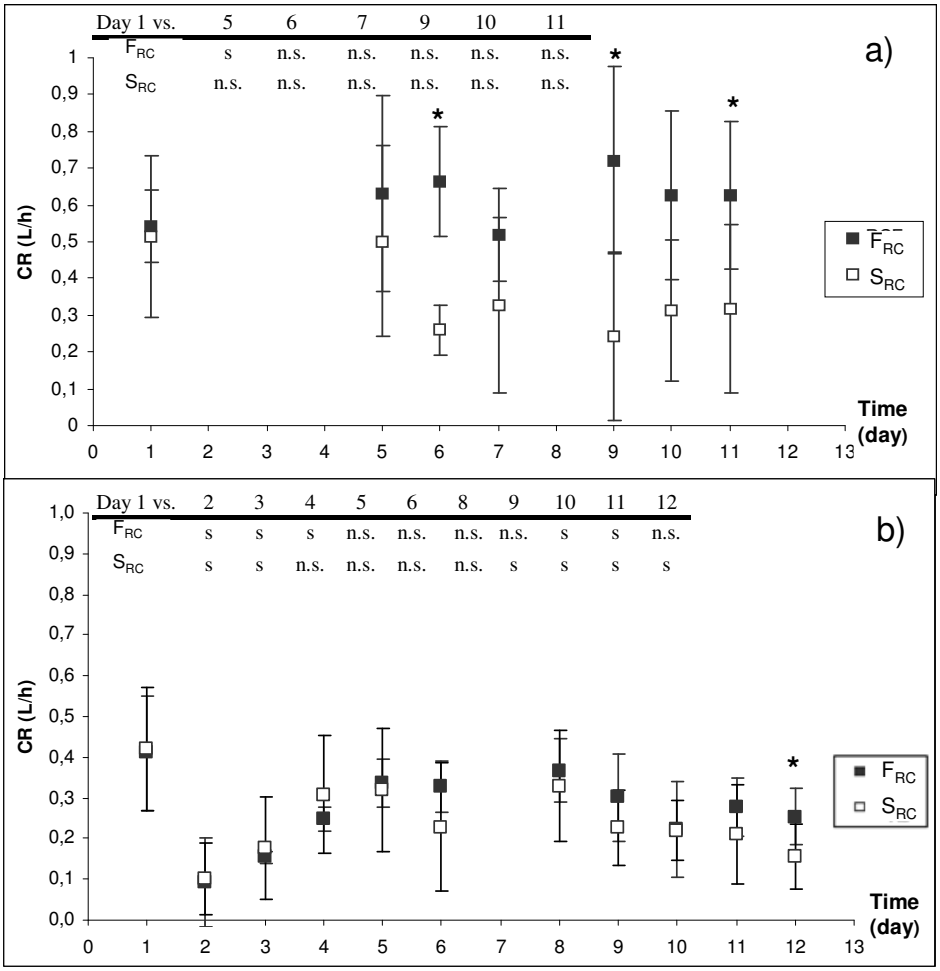

Figure 2.6. Time-course of clearance rate (CR: L/h) of F_{RC} (full squares) and S_{RC} mussels (empty squares) from Treatment II fed H_L (a) and H_H (b) diets. CR of F and S mussels were compared, for every measurement day, using Student´s t-test and significant differences ($p<0.05$) are indicated with an asterisk. Each mussel group daily CR mean values were compared with their respective initial CR values (day 1) by repeated measurements ANOVA (LSD confidence interval adjustment). A summary is shown in the upper part of the figure: s= significant differences ($p<0.05$) and n.s. (non-significant differences, p value>0.05).

H_H diet. The time-course of CR was very similar in F_{RC} and S_{RC} mussels (Figure 2.6b). CR in F and S individuals was 0.041 ± 0.014 L/h and 0.042 ± 0.015 L/h respectively the first day. CR was sharply reduced from the first to the second day of feeding (from ≈ 0.4 to 0.1 L/h) and progressively recovered from days 3 to 5. From day 6 onwards, CR of F mussels remained constant, but from day 9 (for S) and day 10 (for

F), CR was again reduced. The remaining physiological parameters of the energy balance of F and S mussels determined on days 1 and 12 are shown in Table 2.7. On day 1, no significant differences between F_{RC} and S_{RC} mussels were recorded in any of the physiological parameters. During the experimental period of feeding, significant changes of CR, AE, AR and SFG occurred. Irrespective of *growth condition*, CR of mussels was significantly reduced from the first to the last day (12^{th} day) of the experiment (Figure 2.6b). Although AE increased, the resulting absorption rate was found to decrease in the two groups. VO_{2R} was found to significantly increase during the feeding period in F mussels. At day 12 of feeding, F_{RC} mussels displayed significantly higher CRs than S_{RC} mussels (0.27±0.05 and 0.17±0.09 L/h, respectively) but lower absorption efficiencies (0.50±0.15 and 0.63±0.10 in F and S, respectively). Thus, no significant inter-group differences were found in AR or SFG.

Table 2.6. Physiological parameters at day 1 (acute response) and day 11 (acclimated response) of F_{RC} and S_{RC} mussels from Treatment II fed H_L diet. OIR: organic ingestion rate, AE: absorption efficiency, AR: absorption rate, VO_{2R}: routine oxygen consumption and SFG: scope for growth. Significantly different means for F_{RC} and S_{RC} mussels at both acute and acclimated responses are indicated with different letters. Significant ($p<0.05$) differences between acute and acclimated responses in each experimental group (F_{RC} or S_{RC}) are indicated with asterisks.

Physiological parameter	Mussels group	Acute response	Acclimated response	
OIR (mg/h)	F_{RC}	0.27 ± 0.05 [a]	0.28 ± 0.09 [a]	
	S_{RC}	0.25 ± 0.11 [a]	0.14 ± 0.10 [b]	
AE (fraction)	F_{RC}	0.55 ± 0.16 [a]	0.62 ± 0.15 [a]	
	S_{RC}	0.66 ± 0.15 [b]	0.81 ± 0.07 [b]	
AR (mg/h)	F_{RC}	0.14 ± 0.03 [a]	0.15 ± 0.04 [a]	
	S_{RC}	0.16 ± 0.05 [a]	0.11 ± 0.07 [a]	
VO_{2R} (mL/h)	F_{RC}	0.043 ± 0.010 [a]	0.063 ± 0.011 [a]	*
	S_{RC}	0.055 ± 0.021 [a]	0.081 ± 0.017 [a]	*
SFG (J/h)	F_{RC}	1.80 ± 0.56 [a]	1.63 ± 0.58 [a]	
	S_{RC}	1.79 ± 0.85 [a]	0.42 ± 1.46 [a]	

Table 2.7. Same as Table 6 for mussels fed H_H diet

Physiological parameter	Mussels group	Acute response	Acclimated response	
OIR (mg/h)	F_{RC}	0.52 ± 0.18 [a]	0.38 ± 0.08 [a]	
	S_{RC}	0.53 ± 0.19 [a]	0.24 ± 0.12 [b]	*
AE (fraction)	F_{RC}	0.42 ± 0.11 [a]	0.50 ± 0.15 [a]	
	S_{RC}	0.49 ± 0.16 [a]	0.66 ± 0.10 [b]	*
AR (mg/h)	F_{RC}	0.21 ± 0.09 [a]	0.17 ± 0.05 [a]	
	S_{RC}	0.24 ± 0.03 [a]	0.15 ± 0.06 [a]	*
VO_{2R} (mL/h)	F_{RC}	0.050 ± 0.007 [a]	0.068 ± 0.016 [a]	*
	S_{RC}	0.059 ± 0.015 [a]	0.061 ± 0.012 [a]	
SFG (J/h)	F_{RC}	3.03 ± 1.84 [a]	1.84 ± 1.03 [a]	
	S_{RC}	3.18 ± 0.78 [a]	1.65 ± 1.21 [a]	*

Gill area of F_{RC} and S_{RC} mussels

Fast growing mussels (F_{RC}) had significantly higher gill areas than slow growing mussels (S_{RC}) (Figure 2.7). The gill surface area was approximately 17% larger in F_{RC} mussels (mean GA=40 mm^2) than in S_{RC} mussels (mean GA= 33 mm^2).

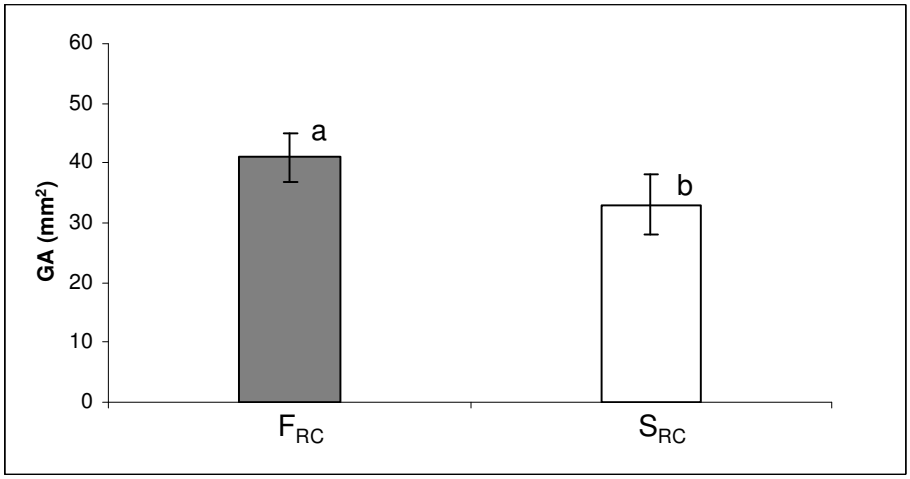

Figure 2.7. Gill surface-area (mm^2) of F_{RC} (solid bars) and S_{RC} (empty bars) mussels selected on Treatment II. Letters indicate significant differences tested by Student´s t-test (p value=0.001)

Discussion

The physiological profiles of fast and slow growing mussels obtained in Treatment I (continuous feeding during discontinuous immersion) and Treatment II (discontinuous feeding during continuous immersion) are substantially different, reinforcing the notion formulated by Tamayo et al. (2016) that trophic characteristics could critically determine the physiological processes underlying fast growth in bivalves. In Treatment I, the feeding rate and possibly pre-ingestive selection were the physiological parameters responsible for size differentiation. At low food concentrations of both high and low quality (i.e., diets H_L and L_L), fast growing mussels (both F_{BP} and F_{AP}) achieved a 2-fold higher scope for growth than slow growing mussels in strict correspondence with their higher clearance rates. The increase in the particle concentration of high-quality food (from H_L to H_H) was found to promote a general reduction of the clearance rate that led to the cancellation of most significant differences in SFG among groups. This same pattern of convergence between growth groups with the high food ration has been previously reported for mussels (Tamayo et al. 2016; Fernández-Reiriz et al. 2016), clams (Tamayo et al. 2011, 2013) and oysters (Tamayo et al. 2014). The CR reduction in response to increasing particle concentration of high organic content has been thoroughly reported in bivalves (since Foster-Smith, 1975) and interpreted as a mechanism allowing the regulation of ingestion rate and gut passage time (Thompson and Bayne, 1974; Bayne et al. 1987, 1988; Navarro et al. 1994). With low-quality diets, increasing particle concentration (from L_L to L_H) promoted a less pronounced reduction of CR in all mussel groups. Such differences in CR behaviour of mussels fed diets of different qualities are consistent with previous findings in bivalves (Bayne et al. 1987; Navarro et al. 1994; Iglesias et al. 1996; Ward and Shumway, 2004). These differences have been explained by the capacity of bivalves to improve the absorption rate at high concentrations of low organic content food, by regulating the ingestion rate by means of rejecting filtered matter via pseudofaeces after a pre-ingestive selection of organically rich particles (Navarro and Iglesias, 1993; Urrutia et al. 1997). The higher ability of F mussels to select organic food items (as indicated by a significant effect of growth condition on SE) might have contributed to the higher SFGs attained by F individuals with the L_H diet. A relevant feature of these differences in SE between F and S individuals is that they were not restricted to mussels reared under high particle concentration (i.e., under conditions

compelling pseudofaeces production) but were also present in mussels grown with low particle concentrations (i.e., under conditions in which pseudofaeces were absent). Thus, it seems that a higher ability for pre-ingestive selection is an inherent feature of fast growing mussels, rather than being an effect promoted by long-term acclimation to the diet. To summarize, the present experiments have shown that, in mussels fed continuously during the immersion phase, and irrespective of the food concentration at which mussels were reared, inter-individual growth potential differences resulting in size-differentiation are caused by the existence of endogenously determined differences in the feeding rate and the efficiency of the pre-ingestive processes for particle selection.

Most studies comparing physiological behaviour of growth groups (Holley and Foltz 1987; Bayne et al. 1999a; Toro and Vergara 1998; Pérez-Camacho et al. 2000; Pace et al. 2006; Tamayo et al. 2011, 2013, 2014; Fernández Reiriz et al. 2016) have reported that endogenous differences of growth potential are caused by a combination of endogenously determined differences in the feeding rate (*acquisition model* as defined by Bayne, 1999) and the energetic costs of metabolic processes (*metabolic efficiency model* as defined by Bayne, 1999). However, the absence of significant differences between the slopes of lines relating routine metabolic rate with absorption rate for F and S mussels indicates that energetic costs of the metabolic processes involved in food processing and growth are similar in fast and slow growers when given per unit of absorbed energy. Thus, recorded innate differences in the capacity to acquire and select food particles (*acquisition model*) remain as the main physiological trait accounting for inter-individual differences in growth potential under the feeding conditions given in Treatment I.

The results obtained with mussels submitted to restrictive nutritional conditions (Treatment II) depicted a very different panorama. To begin with, differences in the capacity to acquire/process food are unlikely causing the growth differences observed between F_{RC} and S_{RC}, given the reduced time at which food was available during the 11 months of the size-differentiation phase. Indeed, the feeding experiments performed with selected fast and slow growing individuals revealed no significant differences in feeding and absorption rates (CR, IR and AR), and SFG values were found between fast and slow growers in the acute (1 day) response to feeding. In contrast, the recorded significant differences in the standard oxygen consumption between F_{RC} and S_{RC} mussels indicate that size-differentiation in mussels submitted to restrictive nutritional

conditions resulted from endogenous inter-individual differences in the capacity to reduce metabolic rate during the period of starvation. F_{RC} specimens had a 33% lower VO_{2S} than S_{RC} mussels. This represents a noticeable difference in energy saving that likely sufficed to cause observed growth-rate differences between F and S mussels, especially after considering the high proportion of time that mussels were submitted to starvation (6 out of 7 days = 85% of the time) during the size-differentiation period. The present results are consistent with those obtained by Tamayo et al. (2016), confirming that endogenous factors contributing to reducing the metabolic costs of maintenance can play a significant role in the size differentiation of mussels. Significant differences in the VO_{2S} (the *allocation model*) have been previously reported to explain differences between selected lines of fast and slow growing oysters *Crassotrea gigas* (Bayne, 1999), and inter-individual growth rate differences in hatchery-reared juveniles of *Crassostrea virginica* (Pernet et al. 2008). However, in both cases, fast growers combined lower standard metabolic rates with faster rates of feeding (i.e., *allocation* plus *acquisition* models). The importance of this physiological mechanism in modulating the innate capacity to grow is further strengthened by the observation that the ability to display a reduced SMR during periods of negative scope for growth has been related with higher degrees of multi-locus heterozygosity (Koehn and Shumway, 1982; Rodhouse and Gaffney, 1984; Diehl and Koehn, 1985; LeBlanc et al. 2008). One feature of such metabolic reduction reported in oysters appears related with the membrane phospholipid composition, particularly their unsaturation index (Pernet et al. 2008), which is in agreement with the proposed role of membranes as metabolic pacemakers (Hulbert and Else, 1999, 2004)

Tamayo et al. (2016) suggested that the size differentiation of juvenile mussels in natural populations might result from divergent growth strategies in contrasting scenarios. Periods of good trophic conditions would maximize growth rates of those individuals that are well equipped to acquire and process food (*fast feeders*), whereas periods of restricted trophic conditions would have a lower adverse impact on those individuals capable to down-regulate VO_{2S}-s (*energy savers*). *Fast feeders* and *energy savers* could be considered to represent differentiated phenotypes of fast growing mussels. However, the feeding experiment performed with F_{RC} and S_{RC} mussels has revealed another relevant feature of the physiological basis underlying fast growth that might alter the perception of the existence of two F phenotypes. The medium-term (12-

day) acclimation of mussels to a continuous food supply, after a long rearing period of discontinuous feeding, was found to promote an increase in the VO_{2R} in both F_{RC} and S_{RC} mussels (both with H_L and H_H diet), attaining statistical significance in F individuals. This increase very likely represents the energetic costs associated with the synthesis of enzymes involved in the reactivation of the depressed digestive machinery, as reported in other studies of starvation and re-feeding in bivalves (Albentosa et al. 1996; Ibarrola et al. 2000; Albentosa and Moyano 2008). The relevant feature distinguishing fast and slow growing mussels was that even though the VO_{2R} increase was similar for both type of mussels, F_{RC} mussels were capable of displaying significantly higher clearance rates by the end of the experiment (11 days or 12 days with H_L and H_H diets, respectively). Our interpretation is that reduced VO_{2S} recorded in F mussels would allow for increased metabolic scope for feeding and growth (MSFG), thus promoting high CR after acclimation to a continuous feeding regime. Hence, fast growing mussels in the scenario represented by Treatment II would result from the capacity of individuals to shift from *energy savers* to *fast-feeders* in response to improved feeding conditions.

The present experiments have shown that endogenous difference in filtering activity (and pre-ingestive selection skills) constitutes pivotal physiological features determining growth potential in bivalves, thus suggesting the existence of genetically determined inter-individual differences in the functional capacities of the feeding organs (gills and palps). Not surprisingly, the present study has shown that F mussels (from both treatments) had significantly larger gill areas than S mussels, which is in good agreement with the recorded differences in feeding rates. Similar differences in gill areas between F and S growing individuals have also been found in clams (Tamayo et al. 2011). Studies analysing inter-specific differences in size-specific clearance rate and gill areas (Ibarrola et al. 2012) and intra-specific allometric scaling of both CR and GA (Meyhofer, 1985; Riisgard, 1988; Jones et al. 1992; Pouvreau et al. 1999) have led to the generally accepted conclusion (Honkoop et al. 2003) that both inter-specific and size related intra-specific differences in clearance rates are explained by corresponding differences in gill surface areas. Furthermore, in accordance with the registered significant effect of *maintenance condition* in the present study, in other studies, gill surface area has been found to respond to exogenous factors, such as particle availability and composition (Theisen, 1977, 1982; Essink et al. 1989; Payne et al.

1995; Honkoop, 2003; Dutertre et al. 2007) and feeding time (Franz, 1993), indicating that gill area is submitted to a considerable phenotypic plasticity. In the present study, despite the differences in the physiological basis explaining fast growing between the Treatments I and II, F mussels were found to possess significantly higher gill-surface area than S mussels in both cases. Thus, we conclude that endogenous factors regulating the expression of morphometric traits of this organ might play a major role in determining inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*. Differential gene expression in gill tissues between F and S mussels is currently being analysed by microarray techniques, and results will be published elsewhere (Prieto et al., in preparation – chapter 4).

In summary, regarding the hypotheses that were formulated in the introduction, the following conclusions were obtained:

a) Size-differentiation in mussels reared under conditions of continuous feeding during discontinuous emersion is based on differences in the feeding rate (*acquisition model*). No significant differences in the increase of metabolic expenditures, as a consequence of higher absorption rates, were found between F and S growers in either Treatment I or II.

b) Endogenous differences in the standard oxygen consumption (VO_{2S}) have been found to contribute to fast growth under conditions of severe food restriction by discontinuous food supply (Treatment II), indicating that the energy saving during periods of starvation resulting from an innate ability to reduce SMR results in a clear advantage for fast growing. However, the capacity to down-regulate SMR does not seem to contribute to the size-differentiation observed in mussels submitted to a feeding restriction consisting of a daily 7 hs of aerial immersion.

c) In mussels fed a diet promoting pseudofaeces production, fast growth is partly achieved by increased selection efficiency. This ability for a better selection of organics appears to be an innate advantage since it occurs also in F mussels selected under feeding conditions below the pseudofaeces threshold. Thus, we propose that the enhanced capacity for particle sorting seems to be a prevalent feature in fast-growing phenotypes.

d) Differences in gill surface area appeared to underlie differences in the feeding rate (*acquisition model*) recorded between F and S mussels selected under Treatment I.

However, same gill area differences were found for F vs. S mussels in Treatment II, even though there were no differences in size differentiation, in this case, related to the differential ability to acquire food. Endogenous factors affecting the gill surface area are candidates to play a major role in determining inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*.

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Additional files

Table 2.A1. Two-way factor ANOVA_testing significant effects of *growth condition* (F or S) and *maintenance condition* (BP or AP) on physiological parameters of mussels when fed the four experimental diets (H_L, H_H, L_L and L_H) of Treatment I.

H_L diet

Source of variation	DF	SS	MS	F	P
CR					
Growth condition	1	0.897	0.897	18.403	<0.001
Maintenance condition	1	0.100	0.100	2.051	0.168
Interaction	1	0.016	0.016	0.318	0.579
Residual	19	0.926	0.049		
OIR					
Growth condition	1	0.012	0.012	2.050	0.168
Maintenance condition	1	0.110	0.110	18.356	<0.001
Interaction	1	0.002	0.002	0.313	0.583
Residual	19	0.114	0.006		
AE					
Growth condition	1	0.002	0.002	0.164	0.690
Maintenance condition	1	0.007	0.007	0.479	0.497
Interaction	1	0.022	0.022	1.537	0.230
Residual	19	0.277	0.015		
AR					
Growth condition	1	0.039	0.039	18.202	<0.001
Maintenance condition	1	0.005	0.005	2.396	0.138
Interaction	1	<0.001	<0.001	0.070	0.794
Residual	19	0.041	0.002		
VO_{2R}					
Growth condition	1	<0.001	<0.001	0.113	0.740
Maintenance condition	1	<0.001	<0.001	0.095	0.762
Interaction	1	<0.001	<0.001	0.291	0.596
Residual	19	0.004	<0.001		
VO_{2S}					
Growth condition	1	0.001	0.001	6.644	0.018
Maintenance condition	1	<0.001	<0.001	0.897	0.355
Interaction	1	<0.001	<0.001	1.468	0.240
Residual	19	0.002	<0.001		
SFG					
Growth condition	1	13.023	13.023	18.362	<0.001
Maintenance condition	1	2.019	2.019	2.847	0.108
Interaction	1	0.147	0.147	0.207	0.654
Residual	19	13.475	0.709		

H_H diet

Source of variation	DF	SS	MS	F	P
CR					
Growth condition	1	0.097	0.097	4.060	0.058
Maintenance condition	1	0.186	0.186	7.824	0.011
Interaction	1	0.063	0.063	2.658	0.120
Residual	19	0.453	0.024		
OIR					
Growth condition	1	0.116	0.116	4.050	0.059
Maintenance condition	1	0.225	0.225	7.822	0.012
Interaction	1	0.076	0.076	2.647	0.120
Residual	19	0.546	0.029		
AE					
Growth condition	1	0.028	0.028	3.226	0.089
Maintenance condition	1	0.001	0.001	0.117	0.737
Interaction	1	0.023	0.023	2.657	0.120
Residual	18	0.155	0.009		
AR					
Growth condition	1	0.030	0.030	4.137	0.057
Maintenance condition	1	0.047	0.047	6.575	0.020
Interaction	1	0.012	0.012	1.710	0.207
Residual	18	0.129	0.007		
VO_{2R}					
Growth condition	1	<0.001	<0.001	1.102	0.307
Maintenance condition	1	0.001	0.001	5.265	0.033
Interaction	1	<0.001	<0.001	0.034	0.856
Residual	19	0.004	<0.001		
VO_{2S}					
Growth condition	1	<0.001	<0.001	0.001	0.980
Maintenance condition	1	<0.001	<0.001	1.092	0.309
Interaction	1	<0.001	<0.001	0.990	0.332
Residual	19	0.006	<0.001		
SFG					
Growth condition	1	8.459	8.459	3.848	0.065
Maintenance condition	1	11.846	11.846	5.389	0.032
Interaction	1	4.676	4.676	2.127	0.162
Residual	18	39.568	2.198		

L_L diet

Source of variation	DF	SS	MS	F	P
CR					
Growth condition	1	1.705	1.705	28.916	<0.001
Maintenance condition	1	0.036	0.036	0.618	0.441
Interaction	1	0.014	0.014	0.234	0.634
Residual	19	1.120	0.059		
OIR					
Growth condition	1	0.197	0.197	28.966	<0.001
Maintenance condition	1	0.004	0.004	0.621	0.440
Interaction	1	0.002	0.002	0.234	0.634
Residual	19	0.129	0.007		
AE					
Growth condition	1	0.129	0.129	10.015	0.006
Maintenance condition	1	0.003	0.003	0.205	0.656
Interaction	1	0.003	0.003	0.196	0.663
Residual	17	0.220	0.013		
AR					
Growth condition	1	0.093	0.093	30.131	<0.001
Maintenance condition	1	0.002	0.002	0.772	0.392
Interaction	1	0.001	0.001	0.322	0.578
Residual	17	0.053	0.003		
VO_{2R}					
Growth condition	1	0.002	0.002	14.388	0.001
Maintenance condition	1	<0.001	<0.001	0.355	0.559
Interaction	1	<0.001	<0.001	0.899	0.356
Residual	18	0.002	<0.001		
VO_{2S}					
Growth condition	1	<0.001	<0.001	0.172	0.683
Maintenance condition	1	<0.001	<0.001	0.747	0.398
Interaction	1	<0.001	<0.001	3.951	0.061
Residual	19	0.002	<0.001		
SFG					
Growth condition	1	24.134	24.134	23.997	<0.001
Maintenance condition	1	1.188	1.188	1.182	0.292
Interaction	1	0.174	0.174	0.173	0.682
Residual	17	17.097	1.006		

L_H diet

Source of variation	DF	SS	MS	F	P
CR					
Growth condition	1	0.096	0.096	1.504	0.237
Maintenance condition	1	0.108	0.108	1.696	0.210
Interaction	1	0.005	0.005	0.084	0.776
Residual	17	1.082	0.064		
SE					
Growth condition	1	0.165	0.165	8.245	0.011
Maintenance condition	1	0.005	0.005	0.235	0.634
Interaction	1	<0.001	<0.001	0.010	0.921
Residual	17	0.340	0.020		
RP					
Growth condition	1	0.029	0.029	3.705	0.073
Maintenance condition	1	0.043	0.043	5.485	0.033
Interaction	1	0.019	0.019	2.443	0.139
Residual	15	0.119	0.008		
OIR					
Growth condition	1	0.077	0.077	3.045	0.099
Maintenance condition	1	0.075	0.075	2.968	0.103
Interaction	1	0.003	0.003	0.134	0.719
Residual	17	0.432	0.025		
AE					
Growth condition	1	0.008	0.008	3.123	0.098
Maintenance condition	1	0.011	0.011	4.355	0.054
Interaction	1	0.001	0.001	0.572	0.461
Residual	15	0.039	0.003		
AR					
Growth condition	1	0.018	0.018	2.070	0.171
Maintenance condition	1	0.018	0.018	1.980	0.179
Interaction	1	0.001	0.001	0.065	0.802
Residual	15	0.133	0.009		
VO_{2R}					
Growth condition	1	<0.001	<0.001	0.342	0.566
Maintenance condition	1	<0.001	<0.001	0.212	0.651
Interaction	1	<0.001	<0.001	0.029	0.866
Residual	17	0.006	<0.001		
VO_{2S}					
Growth condition	1	<0.001	<0.001	0.149	0.705
Maintenance condition	1	<0.001	<0.001	0.019	0.893
Interaction	1	<0.001	<0.001	0.216	0.648
Residual	17	0.003	<0.001		
SFG					
Growth condition	1	24.369	24.369	5.494	0.031
Maintenance condition	1	10.284	10.284	2.319	0.146
Interaction	1	0.633	0.633	0.143	0.710
Residual	15	75.402	4.435		

Table 2.A2. Student´s paired t-test testing significant effect of time in the physiological parameters of F and S mussels from Treatment II when fed H_L and H_H diets.

		Physiological parameter	DF	Mean difference (acute- acclim.)	Std. deviation	Std. error mean	t	p
H_L diet	F	CR	4	-0.085	0.146	0.065	-1.295	0.265
		OIR	4	-0.010	0.063	0.028	-0.368	0.732
		AE	4	-0.040	0.110	0.049	-0.824	0.456
		AR	4	-0.013	0.025	0.011	-1.151	0.314
		VO_{2R}	4	-0.020	0.013	0.006	-3.473	0.026
		SFG	4	0.169	0.533	0.238	0.707	0.518
	S	CR	4	0.193	0.381	0.170	1.132	0.321
		OIR	4	0.110	0.177	0.079	1.396	0.235
		AE	4	-0.147	0.148	0.066	-2.190	0.091
		AR	4	0.046	0.105	0.047	0.975	0.385
		VO_{2R}	4	-0.026	0.018	0.008	-3.260	0.031
		SFG	4	1.381	1.733	0.775	1.782	0.149
H_H diet	F	CR	5	0.141	0.158	0.065	2.184	0.081
		OIR	5	0.137	0.203	0.083	1.650	0.160
		AE	5	-0.035	0.146	0.060	-0.587	0.582
		AR	5	0.043	0.099	0.040	1.076	0.331
		VO_{2R}	5	-0.019	0.015	0.006	-2.984	0.031
		SFG	5	1.191	1.815	0.741	1.607	0.169
	S	CR	4	0.241	0.116	0.052	4.640	0.010
		OIR	5	0.198	0.234	0.095	2.079	0.092
		AE	5	-0.170	0.119	0.049	-3.494	0.017
		AR	4	0.081	0.063	0.028	2.889	0.045
		VO_{2R}	5	-0.001	0.019	0.008	-0.170	0.871
		SFG	4	1.626	1.291	0.577	2.817	0.048

Chapter 3

Physiological basis of inter-individual growth rate differences of the mussel *Mytilus galloprovincialis* reared under different water temperatures

Abstract

Growth differences between individuals reared under identical environmental conditions indicate the existence of genetically determined inter-individual variation in the growth potential of bivalves. Growth heterogeneity relies on the existence of inter-individual differences in the energy balance caused by genetic differences in the capacity either to acquire food or to use metabolic energy for growth and responding to environmental stress. In the preceding chapters it has been shown that the nurture conditions under which inter-individual size-differentiation occurs alters the nature of the physiological components acting on size differentiation of fast versus slow growing individuals. Temperature is one of the main environmental factors determining growth rate in bivalves. The aim of this study was to ascertain if the capacity for acute and chronic compensation of thermal effects on the physiological rates could be a potential trait contributing to the inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*.

Juvenile specimens of the mussel *Mytilus galloprovincialis* of an homogeneous initial shell-length (approximately 10 mm) collected from a rocky intertidal shore were reared in the laboratory under good feeding conditions (continuous phytoplankton supply) but submitted to two different water temperatures: one group of seeds was maintained in warm (20 ºC) and the other in cold (10 ºC) water temperature. Mussels were maintained at these conditions until size differences among individuals occurred (3 and 6 months for warm and cold treatment, respectively) and this allowed us to select fast (F) and slow (S) growing individuals in both treatments. Individuals selected as fast and slow growers in warm treatment (F_{20} and S_{20}) and cold treatment (F_{10} and S_{10}) were exposed to three experimental-temperatures (10, 15 and 20 ºC) and the time-course of

their response in terms of clearance rate (CR: L/h) and routine oxygen consumption (VO_2: mLO_2/h) was monitored.

Experiments of acute temperature change with mussels reared at 20 ºC showed no significant differences in the thermal sensitivity of clearance or metabolic rates between F and S individuals: in both cases, the temperature drop to 15and 10 ºC promoted an immediate reduction in clearance rate and metabolic rate, but a process of compensation started to occur approximately after 1 week of exposure to the new temperature. At 20 ºC, faster growth in F_{20} as compared with S_{20} individuals was caused by their capacity to display higher clearance rates (approximately 2 times higher in F_{20}) in spite of maintaining similar routine and standard oxygen consumption rates.

Experiments with mussels reared at 10 ºC showed a different picture: in response to acute warming (from 10 ºC to 15 and 20 ºC) F_{10} mussels were capable of compensating the thermal effect on CR and VO_2; however, S_{10} individuals could not. At 10 ºC, F_{10} mussels had two times higher CR than S_{10} individuals, but in contrast with warm treatment, F_{10} mussels also had significantly higher routine and standard VO_2 than S_{10} mussels. Indeed, standard VO_2 of S_{10} individuals at 10 ºC was significantly lower than the routine VO_2 displayed by F_{20} and S_{20} mussels after 20 days of acclimation to 10 ºC, indicating that S_{10} mussels failed to compensate at the long-term (6 months of rearing) the thermal effect that mussels reared at 20 ºC were able to overcome in 20 days.

In both treatments, F mussels had higher gill-surface areas than S mussels. We conclude that two significant factors contribute to endogenous inter-individual differences in growth rate: i) the capacity to display an intense filtering activity, which is functionally correlated with the gill-surface area, and ii) the capacity to compensate the temperature effects on filtration and metabolic rate. The second trait seems to have insignificant contribution to the inter-individual size-differentiation in the mussels at warm environments (20 ºC), but explains a great proportion of inter-individual growth rate differences in cold environments (10 ºC). Since S_{10} individuals were inside the percentile 0-20 of the size-distribution of the population, we conclude that at least 20% of the individuals in our mussel population possess ineffective molecular mechanisms for cold acclimation.

Keywords: Fast growing, metabolic rate, temperature, thermal compensation

Introduction

Growth in bivalves is subjected to great variability. The physical and nutritional environment (Askew 1972; Utting 1986), the physiological performance (Bayne et al. 1999a,b) and the underlying genetic expression (Hedgecock et al 1996, Pace et al 2006) are factors that contribute to promote inter-specific and intra-specific differences in growth rate. The availability of food and the temperature are the environmental variables mostly affecting growth and reproduction in bivalves (Mann 1979; Udding et al. 2012). The effects that the nutritional environment exerts on the physiological parameters of the energy balance of bivalves have been profusely analysed in the last decades (see reviews by Ward and Shumway 2004; Bayne 2004). Bivalves adjust their physiological performance, both at the pre-ingestive and digestive levels, to maximize growth under a wide range of food availability (Winter 1973; Foster-Smith 1975; Navarro and Winter 1982; Bayne et al. 1987; Navarro et al. 1991) and consequently, food concentration and quality are the basic parameters used in dynamic growth models for suspension feeding bivalves (Hawkins et al. 2002; Bacher et al. 2003; Gangnery et al. 2003; Agüera et al. 2017). The temperature has a positive correlation with the physiological parameters of the energy balance, promoting higher growth rates with increasing temperatures (Newell et al. 1977; Buxton et al. 1981; Griffiths and Griffiths; 1987). Kang et al. (2016) have recently analysed the combined effect of the food concentration and water temperature on the energy budget of the clam *Ruditapes philipinarum* measuring the physiological parameters of clams fed 24 experimental conditions (4 temperatures x 6 food concentrations). They showed that main physiological parameters determining the energy budget (clearance rate and routine metabolic rate) increased with rising temperature and food availability.

The endogenous factors contributing to the physiological basis of inter-individual growth rate differences in bivalves have also been analysed in experiments of physiological experiments with bivalves of different species and submitted to different feeding conditions (Bayne 1999; Bayne et al. 1999a, b; Toro and Vergara 1998; Toro et al. 2004; Bayne 2004; Tamayo et al. 2011, 2015). Since the physiological variables involved in the size-differentiation between fast and slow growing individuals vary between studies, it seems that rather than obeying to a simple physiological cause, inter-individual differences in growth rate might arise as the consequence of endogenously determined differences in multiple physiological traits. Tamayo et al. (2016) observed

that the traits causing fast growing in the mussel *Mytilus galloprovincialis* differed between experiments: with mussels reared under good feeding conditions, fast growers had significantly higher clearance rates than slow growers; however, with mussels reared under severe feeding restrictions, fast growers displayed significantly lower standard metabolic rates. Prieto et al (2018) (chapter 2) have recently confirmed that the throphic characteristics of the rearing environment might alter the set of physiological traits differing between fast and slow growing mussels and defined two basic phenotypes of fast growing bivalves: fast feeders and energy savers.

Very few studies have analysed the possibility that differential skills for thermal compensation of physiological processes could contribute to inter-individual differences in the growth rate of ectothermic organisms such as bivalves. Early studies by Hawkins (1985) and colleagues (Hawkins et al. 1987) analysed the protein *turnover* and the energy balance of *Mytilus edulis* acclimated to 10 ºC after warming them to 20 ºC. They found that individuals that grew faster had slower protein turnovers that resulted in lower metabolic rates and greater homeostatic properties against temperature increase events. They concluded that i) the capacity for thermal compensation of metabolic rate affects significantly the rate of protein turnover of the individual and ii) faster protein turnover rates limit the scope for activity, thus, resulting in a short thermal range for positive energetic scope for growth. Pernet et al. (2008) analysed the capacity for thermal adaptation of genetically distinct groups of oysters *Crassotrea virginica* that showed differences in growth rate. They analysed the membrane lipid composition and the energy budget after being acclimated to different temperatures (4, 12 and 20 ºC). They found that individuals from fast growing lines displayed lower standard metabolic rates due to their capability to reduce the unsaturation indexes of membrane lipids. Tamayo et al. (2013) measured the short and medium-term physiological response of fast (F) and slow (S) growing clams (*Ruditapes philippinarum)* after cold (10 ºC) and warm (24 ºC) exposure. They found that growth rate in S individuals was limited by the significantly higher thermal dependency of the metabolic expenditures that promoted higher increase of routine metabolic rate at warm temperatures.

The aim of the present study was to ascertain if the capacity for acute and chronic compensation of thermal effects on physiological rates could be a potential trait contributing to the inter-individual growth rate differences in the mussel *Mytilus galloprovincialis*. For that purpose, juvenile mussels were reared at the laboratory at 20

ºC and 10 ºC until clear inter-individual size-differentiation allowed to select fast (F) and slow (S) growing individuals from each rearing temperature. Selected F and S individuals were then used in subsequent experiments to i) compare the physiological profiles of the mussels being selected as F and S growers in different thermal regimes and ii) analyse the acute effects of temperature change on the filtering activity and metabolic rate of F and S individuals.

Material and Methods

Experimental design

Some hundreds of mussel seeds (*Mytilus galloprovincilis*) were collected in an intertidal area of Antzoras (Bizcay, Spain, 43°24'29.1"N; 2°40'51.0"W) in April 2015. At the laboratory, the shell-length of the mussels was determined and 300 individuals of homogenous shell-length and live weight (10.65 ± 0.56 mm and 0.2 ± 0.04 g) were selected. Mussels were divided in two groups of 150 individuals and reared in seawater tanks in the laboratory until clear inter-individual size-differences arose. The rearing conditions were the same for the two groups, but one group was maintained at 20 ºC water temperature while the second group was maintained at 10 ºC. Mussels were fed a high organic content diet (f ≈ 0.7) consisting in a mixture of cells of the microalgae *Isochrysis galbana* (T-iso) and shellfish diet (a commercial mix of four microalgae: *Isochrysis, Pavlova, Tetraselmis* and *Thalassiosira weissflogii*) in a ratio 9:1 respectively, with particles of previously sieved silt. The diet was dosed at an approximated concentration of 1.5 mm^3/L. The tanks were cleaned two times per week. During cleaning, mussels were pulled apart one from each other to avoid inter-individual competition for food. Shell-length was measured with 0.05 mm accuracy caliber and live-weight was determined using a 0.01 mg accuracy balance. Mussels were maintained under these constant conditions until clear inter-individual size differences were found (3 and 6 months for mussels maintained at 20 ºC and 10 ºC respectively). After this period, 60 individuals representing extreme percentiles of size distribution (P_{20} and P_{80}), were selected in each acclimation temperature, to represent fast (F) and slow (S) growers respectively. Accordingly, four experimental groups of mussels were obtained: fast growers selected at 20 ºC (F_{20}), slow growers selected at 20 ºC (S_{20}), fast growers selected at 10 ºC (F_{10}), and slow growers selected at 10 ºC (S_{10}).

Mussels from these four groups were used then in thermal experiments designed to analyse the acute effects of temperature change on physiological performance; selected F and S mussels from warm (F_{20} and S_{20}) and cold treatments (F_{10} and S_{10}) were exposed to three experimental-temperatures (T_{exp}: 10, 15 and 20 ºC) and the time-course of CR and routine VO_2 was monitored. Afterwards, mussels were starved and the evolution of oxygen consumption was analysed until a stable value that was considered to represent standard VO_2 was attained (see Figure 3.1). The difference between routine VO_2 at the onset of starvation and standard VO_2 (the reduction of oxygen consumption during starvation) represents the metabolic scope for feeding and growth (MSFG). The monitoring of CR and routine VO_2 of mussels at their corresponding acclimation temperature (i.e. at 20 ºC for F_{20} and S_{20} mussels and at 10 ºC for F_{10} and S_{10} mussels) was complemented with the collection of water and faeces samples that allowed to compute the complete set of physiological parameters underlying the energy balance.

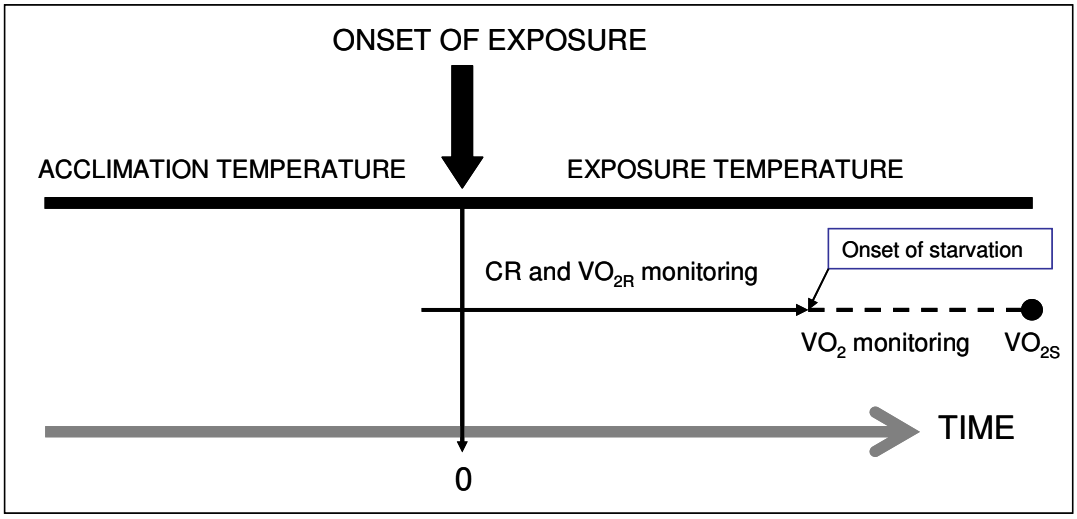

Figure 3.1. Experimental design

Thermal experiments with selected F and S mussels

For determination of the physiological rates, five individuals (n=5) from each mussel group (F_{20}, S_{20}, F_{10} and S_{10}) were placed on filtration chambers. The chambers consisted in borosilicate glass bottles of 150 ml with inflow and outflow lines drilled in the plastic lid. Water from a thermostatic feeding tank containing the experimental diet was recirculated through the chambers by means of multichannel peristaltic pumps

regulated to produce flow rates appropriate to achieve 15-30% reduction in the particle concentration inside the chambers. A mixture of *I.galbana* (T-iso) and silt particles (≈ 2.5:1) was pumped to the feeding tanks from concentrated stocks using peristaltic pumps at rates that were set to provide for a constant concentration of 20,000 particles per ml, (i.e. ≈ 1.5-2 mm^3/l). Particle concentration in the feeding tanks was maintained at stable levels by frequently checking the concentration with a particle counter Coulter Multisizer 3.

Physiological measurements

Clearance rate (CR: L/h) was measured according to the expression of Crisp (1971):

$$CR = F^* ((Ci-C_0)/Ci) \quad ,$$

Where F is the flow rate (L/h), Ci is the particle concentration in the control outflow and C_0 is the particle concentration in the experimental chamber outflow. The concentration of particles was measured using a counter coulter Z1. Daily clearance rate recorded for each individual was the average value of measurements taken every hour for a period of 11-12 hours. Clearance rate was monitored over 8-22 days of exposition period.

Oxygen consumption (VO_2: mL O_2/h). To determine the *routine oxygen consumption* (VO_{2R}) mussels were removed from feeding chambers, and introduced into 150 ml chambers sealed with LDO oxygen probes that were connected to oxymeters (HATCH HQ40d). Rates of VO_2 were derived from the decrease of dissolved oxygen concentration of the water over the time. Oxygen concentration was registered every 5-10 minutes until values decreased 20-30% of initial baseline. A control chamber was used to check the stability of oxygen concentration. When mussels were starved, the oxygen consumption was measured daily, until a stable metabolic expenditure was detected for 2-3 consecutive days that was considered to represent the *standard oxygen consumption* (VO_{2s}).

Energy balance. To analyse the existence of potential differences in the physiological basis underlying inter-individual growth rate differences between mussels reared at different temperatures, the complete set of parameters determining the energy balance was determine in selected F and S individuals at their corresponding rearing temperature (i.e. at exposure temperature of 20 ºC for F_{20} and S_{20} mussels, and at 10 ºC

for F_{10} and S_{10} mussels). For that purpose, water and faeces samples produced by mussels during the feeding period were collected. Water samples were filtered onto ashed pre-weighed GF/C glass-fibber filters and subsequently processed to determine total particle matter concentration (TPM: mg/L) inorganic particulate matter (PIM: mg/L) and organic particulate matter (POM: mg/L). Retained salts were rinsed out with a solution of ammonium formate (0.9 %). TPM and PIM were estimated as the dry and ash weight increment of the filters respectively. POM was calculated as the difference between TPM and PIM. Organic content of food (f) was estimated as f= POM/TPM.

Ingestion rate of organic matter of individual mussels (OIR: mg POM/h) was calculated as OIR = CR * POM

Samples of faeces were filtered onto ashed pre-weighed GF/C glass-fibber filters and processed as described for water samples to determine total matter, inorganic matter and organic matter in the faeces. Organic content of faecal matter (e) was estimated as the ratio of organic/total matter. Then, *Absorption efficiency* (AE: Decimal units) was determined using the Conover (1966) expression:

$$AE= (f-e)/(1-f)*e$$

where f and e represent, respectively, the organic content of food and faeces. Once AE was determined, individual *absorption rate* (AR: mg/h) was calculated as AR = OIR * AE. The resulting *Scope for growth* (SFG: J/h) was estimated as the difference between absorbed energy (AR: J/h) and metabolic expenditure (RMR: J/h). AR (mg/h) was transformed into energetic values (J/h) using an energy equivalence of 18.75 J/mg (Whyte, 1987). Oxygen consumption (VO_2) was transformed into energy values using an oxycaloric coefficient of 20.08 J/mLO_2 (Gnaiger, 1983)

Size-Standardization

Physiological determinations are expressed in terms of live weight. Clearance rate and oxygen consumption were standardized to a common live weight of 1 gr. according to the following expression (Bayne and Newell, 1983):

$$Y_{STD}= (1/W_{EXP})^b * Y_{EXP},$$

where Y_{STD} and Y_{EXP} represent standard and experimental physiological rate respectively, and We the experimental weight. The power value that scales

physiological rates to body weight (b) used for clearance rate and oxygen consumption were 0.58 (Bayne and Hawkins, 1997) and 0.724 (Bayne et al. 1973).

Thermal dependence of physiological parameters

Thermal dependence of physiological rates was determined according to the Van´t Hoff equation:

$Q_{10}= (Y_1/Y_0)^{(10/(T1-T0))}$

Where Y_o is the measured physiological rate at T_0 temperature, and Y_1 the determined one at T_1 temperature.

Gill-surface area (GA: mm^2)

After the experiments, individual mussels were dissected and placed on graph paper for setting the scale. A photograph of the internal tissues of each mussel was taken with a digital camera and the gill-surface area of each individual was calculated using *ImageJ* program. The displayed data correspond to one side of a demibranch. Gill areas were standardized for an equivalent 1 gr live-weight mussel according to the expression:

$$GA_{STD}= (1/W_{EXP})^b * GA_{EXP},$$

where GA_{STD} and GA_{EXP} represent the standardized and experimental gill area, respectively, and W_{EXP} is the experimental live-weight of the mussel. The power function that scales gill area to live-weight was 0.66 (Jones et al. 1992; Vahl, 1973; Hawkins et al. 1992).

Statistical analysis

Significant differences in the growth rate of the mussels during the rearing period at warm (20 ºC) and cold (10 ºC) temperatures were analysed by testing significant differences in the slope of linear regressions (least-square process) of mean shell-length of mussels (Y) vs time (X). The effect of *growth condition* and *time* on the physiological parameters (CR, VO_2) and growth rate was analyzed by repeated measurements two-way ANOVA. *Time* was denoted as *exposure time* when determinations were performed under feeding conditions, (this is, CR and VO_{2R} measurements) and as *starvation time* when VO_2 was measured in starved mussels. Normal distribution of the data was tested using Shapiro-Wilk prior to the statistical

analysis. Data sphericity was tested by Mauchly test. Accordinly, univariate statistical approach (Asumed sphericity test) or multivariate approaches (Pilai´s trace test) was used. Multiple comparison LSD test was used to compare the monitorization data of each physiological parameter.

The effect of *growth condition* and *acclimation temperature* factors in gill-surface area and in the physiological parameters determining the energy budget of the mussels at their rearing temperatures (F_{20} and S_{20} at 20 ºC and F_{10} and S_{10} at 10 ºC) were analyzed with two-way factor ANOVA. Homogeneity of variances was checked with Levenne test, and accordingly, Games-Howell or Tukey test was applied for the multiple comparisons. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp. Released 2010. Armonk, NY: IBM Corp.). Comparison of slopes and elevations between linear regressions were performed following the ANCOVA procedures described in Zar (2010).

Results

The growth rates of experimental mussels reared at acclimation temperatures of 20 ºC and 10 ºC were computed by adjusting mean shell-length values to linear regression models. The resulting equations are as follows:

20 ºC: GR= 0.093 (±0.002) *day + 10.653 (±0,073), F=2020.3, p<0.0001
10 ºC: GR= 0.035 (±0.001) *day + 10.237 (±0,059), F=1992.5, p<0.0001

Significant differences between slopes and elevations were found between 20 ºC and 10 ºC mussels in the ANCOVA analysis (Slope test: t=14.28, df=1, 14, p<0.05; elevation test: t=10.05, df=1, 8, p<0.05). Thus, growth rate of mussels grown at 20 ºC was nearly three times higher than that in mussels reared at 10 ºC. Accordingly, the selection of fast (F) and slow (S) growing individuals was performed earlier (60 vs 150 days) in the warm treatment (20 ºC) than in the cold treatment (10 ºC). Table 3.1 shows the body size (shell-length and live weight) and growth rates (mm/d) of selected fast and slow growing individuals from the two different acclimation temperatures (F_{20} and S_{20} in the warm treatment and F_{10} and S_{10} in the cold treatment). F individuals grew significantly faster and attained approximately 100% more weight and 50% more length than S individuals, in both acclimation temperatures. Growth rate of mussels was significantly higher at 20 ºC than at 10 ºC.

Table 3.1. Shell-length (L: mm), live weight (W: g) and growth rate (GR: mm/day) of mussels selected as fast and slow growers (n=15) after 60 and 150 days of maintenance at 20 ºC and 10 ºC respectively (mean values ± SD).

	W (mg)	L (mm)	GR (g/day)	GR (mm/day)
F_{20}	0.8 ± 0.11	18.6 ± 0.8	0.011 ± 0.002	0.135 ± 0.013
S_{20}	0.4 ± 0.07	13.2 ± 1.1	0.003 ± 0.001	0.045 ± 0.014
F_{10}	0.9 ± 0.12	19.4 ± 1.0	0.005 ± 0.001	0.060 ± 0.007
S_{10}	0.3 ± 0.06	11.9 ± 0.8	0.001 ± 0.001	0.011 ± 0.005

Monitoring of the clearance rate and the oxygen consumption of F and S mussels at different exposure-temperatures.

Mussels reared at 20 ºC

Mussels reared at 20 ºC at T_{exp} of 20 ºC.

Figure 3.2a and 3.2b show the clearance rate and oxygen consumption, respectively, of F_{20} and S_{20} at 20 ºC. A summary of the repeated measurements two-way ANOVA testing significant effects of *growth condition* (F vs S) and *exposure time* on physiological parameters is shown in Table 3.2. F_{20} mussels had significantly higher CR than S_{20} mussels *(growth condition* exerted significant effect on the CR, Table 3.2) and no significant effect of *exposure time* or the interaction term on the CR was registered. Mean CR values for F_{20} and S_{20} mussels were 0.46 ± 0.11 and 0.25 ± 0.07, respectively. Routine VO_2 was measured in three occasions (Figure 3.2b). No significant differences were recorded and mean VO_{2R} for F_{20} and S_{10} were 0.067 ± 0.006 and 0.064 ± 0.002, respectively. Starvation promoted a 35% VO_2 decrease in both groups of mussels, and thus, *starvation time* was found to affect significantly the oxygen consumption during starvation period.

Mussels reared at 20 ºC: response to cooling at 15 ºC

When F_{20} and S_{20} mussels were exposed to 15 ºC, a considerable reduction of CR was registered (Figure 3.2c) in comparison with the CR values recorded at 20 ºC. Both growth groups had statistically the same CR (approximately 0.1-0.2 L/h) during the first 6 days after the exposure to 15 ºC. At day 7, CR of both groups started to increase, however, F_{20} mussels increased their CR (0.558 ± 0.220 L/h at day 11) more than their

slow growing counterparts (0.264 ± 0.081 L/h at day 11), resulting in significant differences between groups in the last 3 days of experiment ($p<0.05$). Accordingly, *growth condition, exposure time* and the interaction of both factors affected significantly the CR of mussels (Table 3.2). Although no initial values were measured (day 0), the CRs of both F_{20} and S_{20} the last days of the experiment were similar to the registered CRs in mussels at 20 ºC.

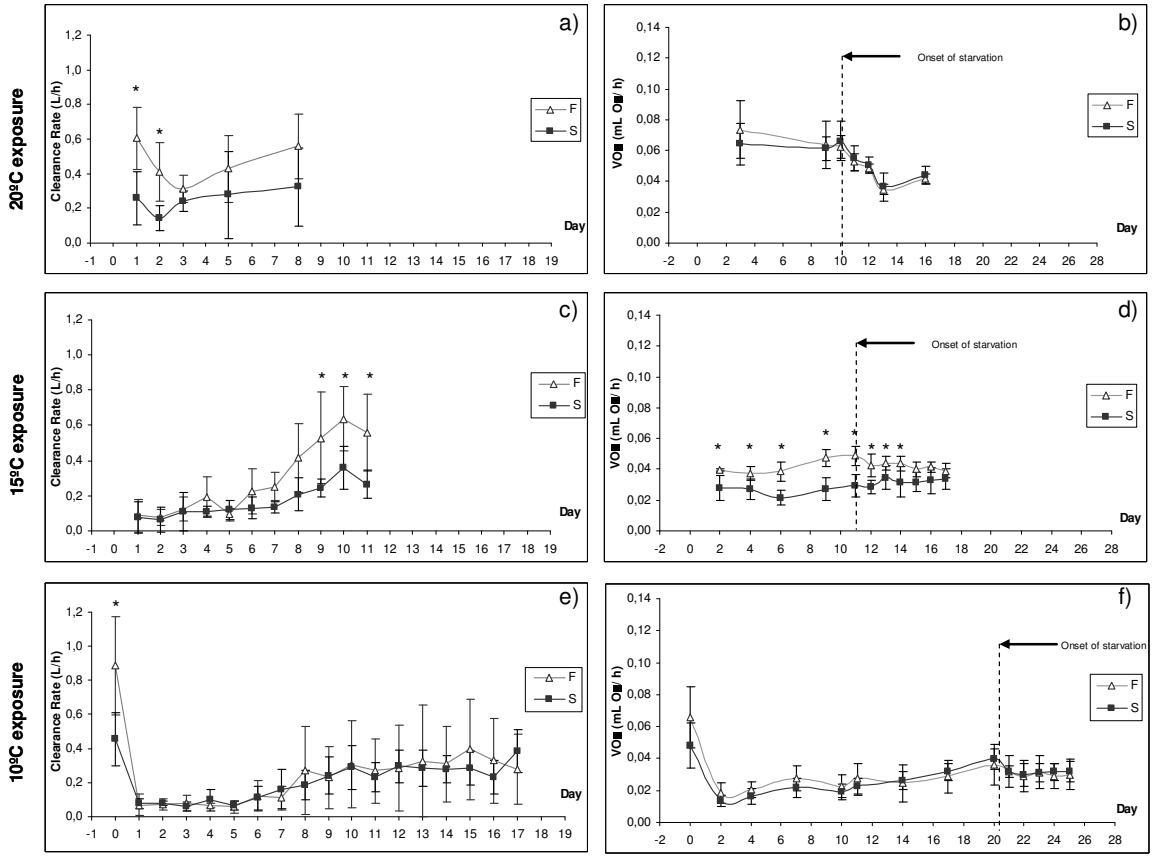

Figure 3.2. Time course of the clearance rate (a, c, e) and the oxygen consumption (b, d, f) of mussels reared at 20 ºC at the three exposure temperatures (20 ºC, 15 ºC and 10 ºC).

Routine VO_2 in both F and S individuals decreased in comparison with the registered VO_{2R} at 20 ºC (Figure 3.2d). Mean values of routine VO_2 were significantly higher in F_{20} than in S_{20} individuals during all the exposure period (*growth condition* had significant effect, Table 3.2). Additionally, F_{20} and S_{20} individuals showed different patterns of VO_2 variation: In F individuals, VO_{2R} increased from 0.039 ± 0.001 (day 1) to 0.049 ± 0.006 mL O_2/h (day 11), but in S mussels VO_{2R} was kept unchanged:

0.028 ± 0.008 (day 1) and 0.029 ± 0.007 mL O_2/h (day 11). Accordingly, *exposure time* and the interaction term exerted significant effect on the VO_{2R} of 20 ºC mussels exposed to 15 ºC (Table 3.2). Starvation did not promote a significant reduction in the oxygen consumption, and thus, only *growth condition* exerted significant effect upon standard VO_2.

Table 3.2. Summary of repeated measurements two-way factor ANOVA testing the significant effect of *growth condition* (F or S) and *exposure time* (Day) on the clearance rate, routine oxygen consumption and oxygen consumption after starvation of mussels reared at 20 ºC

	CR	VO_{2R}	VO_2 after starvation
10 ºC exposure			
Growth condition	0.834	0.493	0.744
Exposure time	**<0.001**	**<0.001**	**<0.001**
Interaction	0.919	**0.035**	0.850
15 ºC exposure			
Growth condition	**0.041**	**0.001**	**0.014**
Exposure time	**<0.001**	**<0.001**	0.262
Interaction	**<0.001**	**0.012**	0.212
20 ºC exposure			
Growth condition	**0.010**		0.652
Exposure time	0.231	n.d.	**<0.001**
Interaction	0.448		0.931

Mussels reared at 20 ºC: response to cooling at 10 ºC

Immediately before temperature change (day 0), F_{20} mussels had significantly higher CR than S_{20} mussels (Figure 3.2e). After temperature change to 10 ºC, the CR of both mussel groups was severely inhibited (reduced to ≈ 0.07 L/h). Mussels started to increase their CR between the 7th and 10th day, reaching ≈ 0.3 L/h at the end of the experiment. No significant differences were found between F_{20} and S_{20} mussels. Thus, the analysis of variance showed a significant effect of *exposure time* and no significant effect of *growth condition* (Table 3.2). Regarding the oxygen consumption, (Figure 3.2f), the exposure to 10 ºC resulted in a reduction of more than 70% in the VO_2 irrespective of growth condition. After that, the VO_{2R} of both F_{20} and S_{20} mussels increased. Accordingly, *exposure time* exerted significant effect on the routine VO_2. The metabolism increased more in S individuals (from 0.013 ± 0.004 to 0.039 ± 0.006 mL O_2/h) than in F individuals (from 0.019 ± 0.006 to 0.036 ± 0.013 mL O_2/h), and thus,

the interaction (*exposure time*growth condition*) had significant effect (p = 0.035 in Table 3.4). After starvation, only *starvation time* was found to exert significant effect on the VO_2 because the decrease of standard metabolism was similar in both mussel groups.

Mussels reared at 10 ºC;

Mussels reared at 10 ºC, at T_{exp} of 10 ºC.

Figure 3.3a and 3.3b show the CR and VO_2, respectively of mussels grown at 10 ºC maintained at 10 ºC. A summary of the repeated measurements two-way ANOVA testing significant effects of *growth condition* (F vs S) and *exposure time* on physiological parameters is shown in Table 3.3. Clearance rates were significantly higher in F_{10} (Mean CR= 0.384 ± 0.100 L/h) than in S_{10} mussels (Mean CR= 0.141 ± 0.070 L/h), and thus, *growth condition* was found to exert significant effect (Table 3.3). A slight but significant temporal change in the CR was observed (*exposure time* was found to exert significant effect). Regarding routine VO_2, no significant temporal changes were recorded and F_{10} mussels had significantly higher than S_{10} mussels (Mean VO_{2R} = 0.053 ± 0.002 and 0.020 ± 0.001 mL O_2/h, respectively) thus, only *growth condition* exerted significant effect (Table 3.3). During starvation, F_{10} mussels reduced significantly oxygen consumption, however, VO_2 of S_{10} individuals did not change significantly (*growth condition* and *starvation time* had significant effect, Table 3.3).

Mussels reared at 10 ºC: response to warming at 15 ºC

The response of the CR after warming water temperature to 15 ºC was very different in F_{10} and S_{10} individuals. In F_{10} mussels, CR increased during the first 3 days (from 0.378 ± 0.120 at day 0 to 0.828 ± 0.085 L/h; i.e. a Q_{10} value of 4.79) and then a compensatory reduction occurred until day 8 (0.198 ± 0.136). Then, CR was maintained at similar value until the end of the experiment the 16th day. On the contrary, CR of S_{10} mussels increased continuously during all the feeding period (from 0.157 ± 0.026 to 0.433 ± 0.131 L/h, Q_{10} 0f 7.58); therefore, F_{10} mussels had significantly higher CR than S_{10} individuals only until the 5th day. Then, due to compensatory reduction of CR in F_{10} and the increase of CR in S_{10} mussels, the differences in CR was inverted in favour of S_{10} individuals (significant differences were found at days 9,10,12,13 and 14).

Accordingly, *exposure time* and the interaction term exerted significant effect on the CR of the mussels, and no effect of *growth condition* was measured (Table 3.3).

Warming to 15 ºC promoted a sudden and intense increase of routine VO_2 in both F_{10} and S_{10} (Figure 3.3d). In F_{10} mussels, VO_{2R} increases from 0.048 ± 0.007 to 0.089 ± 0.009 mL O_2/h at day 4 (which represents a Q_{10} of 3.43), whereas in S_{10} mussels VO_{2R} increases from 0.026 ± 0.010 to 0.053 ± 0.013 mL O_2/h at day 4 (which represents a Q_{10} of 4.15). F_{10} mussels maintained significantly higher routine VO_2 than S_{10} until day 6. After that, in good correspondence with the trend showed by CR, routine VO_2 decreased in fast growers (Q_{10} between last day of feeding and initial day = 1.77). Since VO_{2R} of S mussels was keep at constant values, significantly higher oxygen consumptions were recorded in S_{10} individuals at the end of the feeding period. Accordingly, *exposure time* and the interaction term exerted significant effects on routine VO_2. Starvation promoted a similar decrease in the VO_2 in both mussels groups and thus, only *starvation time* affected significantly standard VO2.

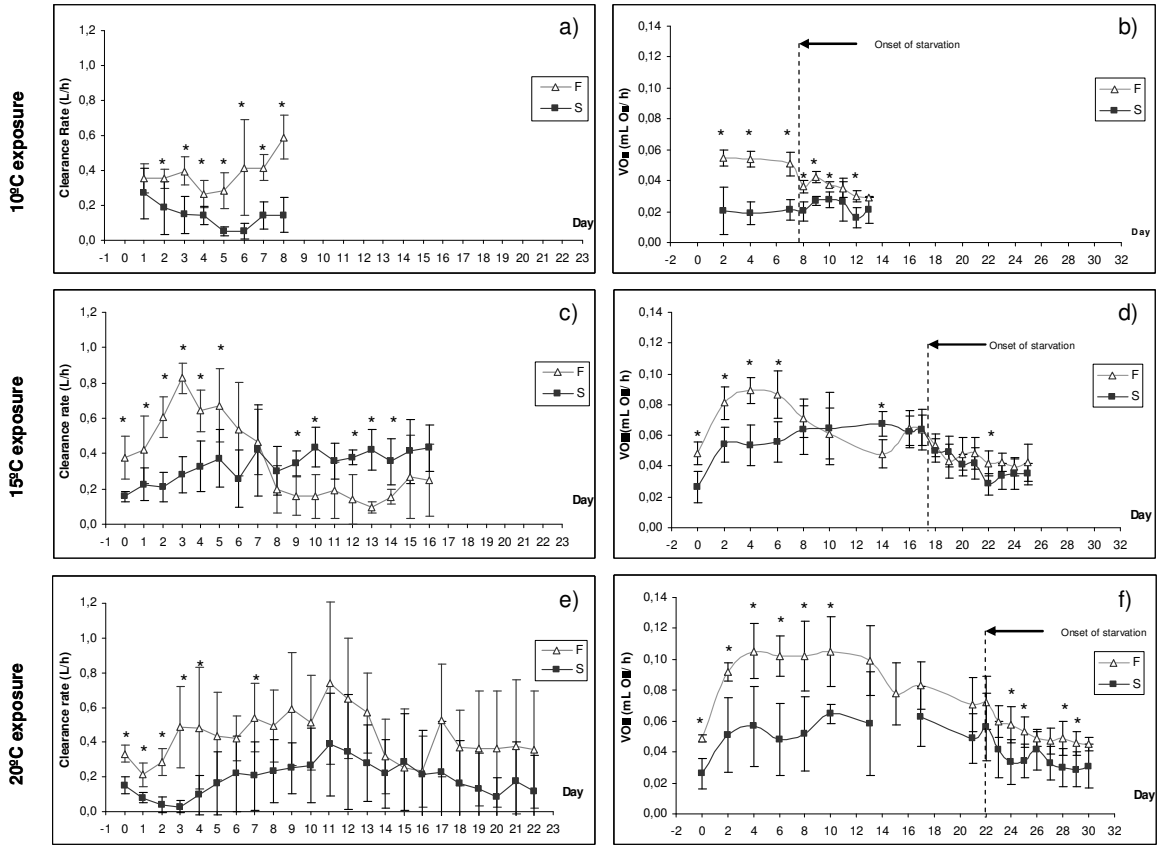

Figure 3.3. Time course of the clearance rate (a, c, e) and the oxygen consumption (b, d, f) of mussels reared at 10 ºC at the three exposure temperatures (20 ºC, 15 ºC and 10 ºC).

Mussels reared at 10 ºC: response to warming at 20 ºC

As an initial response to warming, mussels reduced their CR in comparison with day 0 (Figure 3.3e). After that, CR showed an increasing trend until day 11. Although CR values of F_{10} mussels were higher during all the experiment, differences were significant only the first 4 days and at day 7. Although intra-group variability of CR increased markedly from day 4, *growth condition* exerted significant effect on the CR. *Exposure time* was also found to affect significantly the CR, but no effect of the interaction term was found.

In contrast to the trend exhibited by CR, the initial response to warming consisted in a significant increase of routine VO_2 (Figure 3.3f) in both F_{10} and S_{10} mussels. By day 4, VO_{2R} values doubled those measured at day 0: for F_{10} it went from 0.049 ± 0.002 to 0.105 ± 0.018 mL O_2/h (Q_{10}=2.14), and for S_{10} VO_2 raised from 0.026 ± 0.010 to 0.057 ± 0.026 mL O_2/h (Q_{10}= 2.19). Oxygen consumption decreased in F_{10} mussels to 0.072 ± 0.017 mL O_2/h by the end of the feeding period (Q_{10} between last day of feeding and initial day = 1.77). Both *growth condition* and *exposure time* exerted significant effect on the routine VO_2 of the mussels. The onset of starvation promoted the decrease of VO_2 in F_{10} (from 0.72 mL O_2/h to 0.56 mL O_2/h) and in S_{10} (from 0.41 mL O_2/h to 0.30 mL O_2/h) mussels. F_{10} mussels displayed significantly higher standard VO_2 during most of the starvation period. G*rowth condition, starvation time* and the interaction term exerted significant effect on the standard VO_{2S}.

Table 3.3. Summary of repeated measurements two Factor ANOVA testing the significant effect of *growth condition* (F or S) and *exposure time* (Day) on the clearance rate, routine oxygen consumption and oxygen consumption after starvation of mussels reared at 10 ºC.

	CR	VO_{2R}	VO_2 after starvation
10 ºC exposure			
Growth condition	**<0.001**	**<0.001**	**<0.001**
Exposure time	**0.027**	0.878	**0.049**
Interaction	0.059	0.771	0.069
15 ºC exposure			
Growth condition	0.673	0.097	0.247
Exposure time	**<0.001**	**0.010**	**<0.001**
Interaction	**<0.001**	**0.001**	0.121
20 ºC exposure			
Growth condition	**0.014**	**0.004**	**0.031**
Exposure time	**0.002**	**0.038**	**<0.001**
Interaction	0.849	0.350	**0.035**

Acute thermal effect on metabolic scope for feeding and growth

In the Figure 3.4, we have plotted the mean values of routine VO_2 (recorded immediately before starvation) and the standard VO_2 (recorded at the end of starvation period) in the four experimental mussel groups ($F_{20,}$ S_{20}, F_{10} and S_{10}) at the tested experimental temperatures (10 ºC, 15 ºC and 20 ºC). The figure illustrates the differential effects that short-term changes in exposure temperature promoted on both metabolic rates in fast and slow growing mussels and the resulting metabolic scope for feeding and growth (MSFG, computed as the difference between VO_{2R} and VO_{2S}). The Q_{10} values for standard and routine oxygen consumptions in the range of 10º-20º of exposure temperatures are indicated in the figure. The figure shows that irrespective of the rearing temperature, fast growing mussels displayed higher metabolic scopes for feeding and growth in the range of temperatures tested: in S_{10} and S_{20} mussels, the MSFG is virtually cancelled at low temperatures.

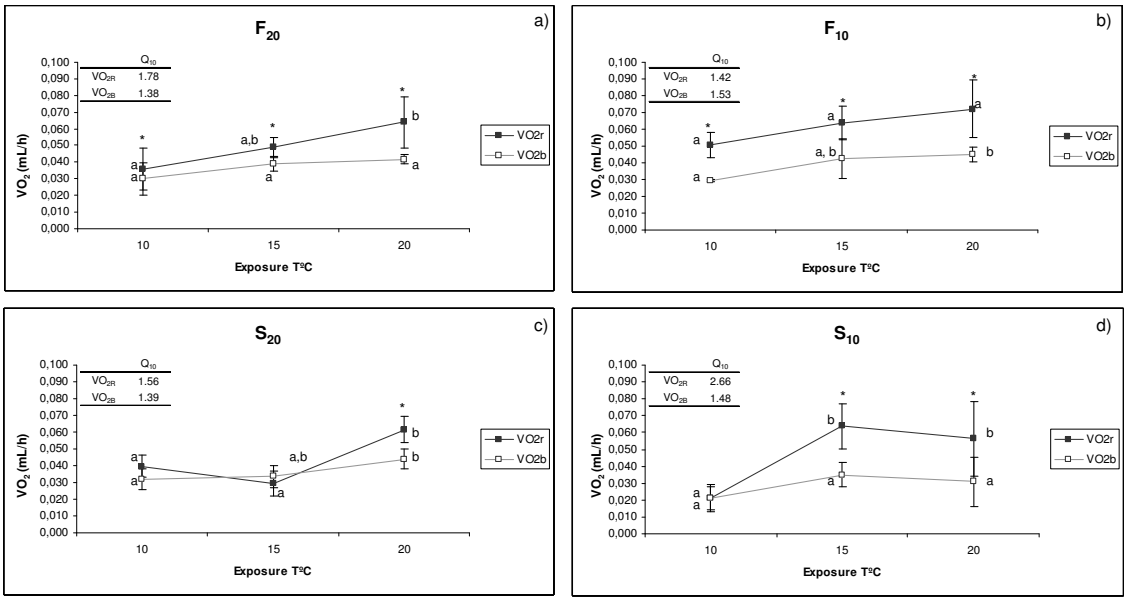

Figure 3.4. Routine oxygen consumption (VO_{2R}) and standard oxygen consumption (VO_{2S}) of F_{20}(a), F_{10}(b), S_{20}(c) and S_{10}(d) mussels at exposure temperature of 10 ºC, 15 ºC and 20 ºC temperatures. At the upper side of each graph, the Q_{10} values for standard and routine oxygen consumption in the range 10-20 are shown.

Energy balance of fast and slow growing mussels reared at 20 and 10 ºC.

During the feeding period of the experiments described above, we collected samples of suspension and faeces produced by F_{20}, $S_{20,}$ F_{10}, and S_{10} mussels exposed to their respective rearing temperatures, in order to compute POM and AE. This procedure allowed us to compute the complete energy balance of the four mussel groups departing from the CR and VO_{2R} values plotted in figures shown in Figures 2 and 3. The resulting physiological components of the energy balance are shown in Table 3.4 together with the gill-surface area measured for the 15 individuals from each mussel group. Significant effects of *growth condition* and *acclimation temperature* on the physiological components of the energy balance were analysed by performing two-way analysis of variance. A summary of the ANOVA is shown in Table 3.4.

Table 3.4. Physiological parameters determining the energy budget and gill surface-area (GA: mm^2) in 10ºC and 20ºC mussels at their maintenance temperatures. CR: clearance rate (L/h), AE: absorption efficiency (fraction), AR: absorption rate (mg/h), VO_{2R}: routine oxygen consumption (mL/h), VO_{2S}: standard oxygen consumption (mL/h), MSFG: metabolic scope for feeding and growth (mL/h) and SFG: scope for growth (J/h). A summary of the p-values of two-way factor ANOVAs testing significant effects of growth condition (F or S) and acclimation temperature (20 ºC or 10 ºC) on the physiological parameters is shown in the right side of the Table. Letters indicate statistical differences between mussel groups.

	Mussel group				Analysis of variance		
	F_{20}	S_{20}	F_{10}	S_{10}	*Growth Condition*	*Acclimation temperature*	*Interaction*
CR (l/h)	0.460 ± 0.079 [a]	0.246 ± 0.075 [b]	0.384 ± 0.063 [a]	0.140 ± 0.072 [b]	**<0.001**	**0.013**	0.650
AE (Fraction)	0.556 ± 0.012 [a]	0.567 ± 0.029 [a]	0.426 ± 0.026 [b]	0.387 ± 0.043 [b]	0.510	**0.001**	0.261
AR (mg/h)	0.190 ± 0.033 [a]	0.104 ± 0.032 [b]	0.095 ± 0.016 [b]	0.031 ± 0.016 [c]	**<0.001**	**<0.001**	0.328
VO_{2R} (ml/h)	0.069 ± 0.009 [a]	0.063 ± 0.005 [a,b]	0.053 ± 0.004 [b]	0.020 ± 0.006 [c]	**<0.001**	**<0.001**	**<0.001**
VO_{2S} (ml/h)	0.042 ± 0.003 [a]	0.044 ± 0.006 [a]	0.030 ± 0.001 [b]	0.021 ± 0.008 [b]	0.204	**<0.001**	**0.035**
MSFG (ml/h)	0.027 ± 0.007 [a]	0.019 ± 0.006 [a]	0.024 ± 0.004 [a]	-0.001 ± 0.008 [b]	**<0.001**	**0.001**	**0.015**
SFG (J/h)	2.185 ± 0.528 [a]	0.683 ± 0.548 [b,c]	0.717 ± 0.233 [b]	0.183 ± 0.248 [c]	**<0.001**	**<0.001**	**0.020**
GA (mm^2)	43.08 ± 5.93 [a]	32.94 ± 6.58 [b]	39.36 ± 5.52 [a]	30.68 ± 7.77 [b]	**<0.001**	**0.007**	0.508

All parameters without exception were significantly higher at 20 ºC than at 10 ºC, thus, *acclimation temperature* exerted a positive significant effect upon all the parameters. Growth condition exerted a significant effect upon all the physiological variables except absorption efficiency and standard metabolic rate. F mussels had significantly higher clearance rate, absorption rate and gill areas than their S counterparts both at 20 and 10

ºC acclimation temperatures, thus, significant effects of *growth condition* and *acclimation temperature* but no effect of the interaction term were obtained in the ANOVA for these parameters. However, the interaction term appeared as a significant factor affecting the metabolic parameters and SFG, indicating the existence of distinct patterns of inter-group differences at different acclimation temperatures. Indeed, the post-hocs analysis indicate that significant differences between F and S mussels in VO_{2R} occurred only in mussels reared at 10 ºC. In other words, the differences in routine metabolic rate (and hence, in SFG) between F and S individuals were comparatively higher in mussels reared at 10 ºC. A similar pattern was also observed for the standard metabolic rate (interaction term is significant in the ANOVA): inter-group differences were higher in mussel reared at 10 ºC, due to the reduced VO_{2S} of S_{10} mussels.

Discussion

The growth rate difference between mussels reared at the laboratory at 20 and 10 ºC (0.093 and 0.035 mm/d, respectively) indicates an intense thermal dependence of growth process in our mussel population (Q_{10}= 2.65). This is not surprising because positive correlations between growth rate and temperature in bivalves have been reported in many occasions (Widdows 1978; Bayne et al. 1983; MacDonald and Thompson 1985; Beiras et al. 1995). The physiological parameters of our mussels reared at 20 and 10 ºC shown in Table 3.4 clearly illustrate the energetic basis for faster growing at higher temperatures: rising temperatures exerted a combined positive effect on filtering activity and absorption efficiency in mussels that was more intense than the thermal effect upon their routine metabolic rate. More interestingly, present results show that fast growing individuals reared at 10 ºC (F_{10}) grew slightly faster than slow growing individuals reared at 20 ºC (S_{20}) (Table 3.1) indicating that endogenously determined inter-individual differences in growth potential exert, as recognize in many studies (Bayne et al. 1999a, b; Tamayo et al. 2011), an outstanding contribution to the intra-population variability in size distribution.

Fast growing individuals displayed significantly higher clearance rates than their slow growing counterparts at both acclimation temperatures. Thus, present results are in good agreement with previous studies showing that filtration rate is the main

physiological function determining faster growing (Holley and Foltz 1987; Bayne et al. 1999a; Toro and Vergara 1998; Pérez-Camacho et al. 2000; Pace et al. 2006; Tamayo et al. 2011, 2013, 2014; Fernandez-Reiriz et al. 2016; Prieto et al. 2018 – chapter 2). In good correspondence with our previous studies (Tamayo et al. 2011; Prieto et al. 2018 - chapter 2), higher filtration rates in fast growing mussels were accompanied by the possession of significantly higher gill-surface areas than slow growers.

Present study shows that rearing (or acclimation) temperature promotes a differential pattern of inter-individual differences in metabolic performance. At 20 ºC, no significant differences in routine or standard metabolic rates and metabolic scope for feeding and growth were found between fast and slow growing individuals, suggesting that the possession of higher clearance rate and gill-surface area was the main physiological feature promoting inter-individual differences in growth potential. However, at 10 ºC, in addition to reduced CR and GA, slow growing individuals had lower routine metabolic rates (standard metabolic rate was also very closed to be significantly lower) and MSFG than fast growers. In ectothermic animals, a temperature decrease induces the reduction of the metabolic rate and the attenuation of physical activity due to the decrease in enzyme activity and the ordering effect in membrane phospholipids, which can lead to membrane dysfunction. At critical low temperatures, severe reduction of mitochondrial, ventilatory and circulatory activities causes a respiratory limitation that promotes an organismic mismatch between oxygen delivery and oxygen demand (Portnet 2001, 2002; Anestis 2010). Such oxygen limitation triggers anaerobic metabolism and the collapse of the physiological functions (Sommer et al. 1997; Frederick and Portnet 2000; Portner 2001). In the temperature-tolerance range, bivalves might compensate this thermal effect by means of tissue-specific homeokinetic (Prosser, 1991) mechanisms that include i) changing the concentration of metabolic enzymes (quantitative changes), (Buckley et al. 2001; Lesser and Kruse 2004; Fields et al. 2012), ii) induction of enzymes with different kinetic properties (qualitative changes) or, more typically, iii) remodelling membrane lipids (homeoviscous adaptation) (Somero 2010; Portner 2010; Pernet et al. 2006, 2007, 2008). The time-course and the degree of compensation of clearance rate and oxygen consumption in bivalves has been the object of considerable debate. Early studies by Widdows and Bayne (1971) shown that, within the temperature-tolerance range, *Mytilus edulis* completely counteracts the acute thermal effects in both CR and VO_2 within 14 days.

Later results have reported that acclimation of clearance rate achieved faster or higher compensation-levels than oxygen consumption (Cusson et al. 2005; Dunphy et al. 2006; Resgalla et al. 2007; but see Kittner and Riisgard, 2005), suggesting that the preservation of the gill function is an outstandingly important feature of the thermal adaptation in bivalves.

In the present study, we have shown that inter-individual differences in the temperature compensation mechanism contribute significantly to the existence of inter-individual differences in growth potential. Slow growing individuals selected at low temperature (S_{10}) showed an extremely reduced ability for chronic thermal acclimation. This differential trait of S_{10} mussels is evidenced by comparing their behaviour with that shown by F_{20} and S_{20} mussels in response to cooling to 10 ºC: both F_{20} and S_{20} reduced dramatically their VO_{2R} and CR (to approximately 0.020 mLO_2/h and 0.10 L/h, respectively) after being transferred to cold water (10 ºC); however, after 20 days of acclimation to the new thermal regime, mussels in both group were able to increase oxygen consumption (0.040 mLO_2/h) and clearance rate (0.3-0.4L/h) and achieved considerably higher levels than those of S_{10} mussels (0.021 mLO_2/h and 0.14L/h, see Table 3.4). In other words, S_{10} mussels failed to compensate at long-term (6 months of rearing) the thermal effect that mussels reared at 20 ºC were able to overcome in 20 days. Moreover, whereas both F_{20} and S_{20} attained positive metabolic scope for feeding and growth after 20 days of acclimation to 10 ºC, S_{10} mussel had a nearly null MSFG, suggesting that the capacity to supply O_2 to the whole organism remained limited by the cold exposure in S_{10} mussels, and thus, the energy for maintenance and/or cost of feeding needed to be obtained by alternative pathways, restricting the organism capacity to acquire food and grow.

Even more, the warming experiments performed in present study with mussels reared at 10 ºC also evidenced that, in contrast with fast growers (F_{10}), mussels selected as slow growers in cold environment (S_{10}) lack the mechanisms for acute thermal compensation. The elevation of 5 degrees (from 10 ºC to 15 ºC) promoted a short-term increase in CR and VO_{2R} of both mussel groups; fast growers were able to compensate thermal effect by day 17, a time course that seems to be consistent with a homeoviscous adaptation response. In contrast, slow growing mussels (S_{10}) were found to increase CR and VO_2 (from 0.2 L/h to 0.4 L/h and from 0.025 to 0.070 mLO_2/h at day 16 respectively) and showed no signs of compensation after 16 days of exposure.

Similarly, a differential behaviour was observed after warming S_{10} and F_{10} mussels up to 20 ºC: in both cases, an initial increase in routine oxygen consumption coincides with a significant reduction in CR that suggests that thermal stress elevate the metabolic rates to levels that surpass the aerobic capacity of mussels. In F_{10} mussels, VO_{2R} increment from 0.049 mL/h to 0.092 mL/h by the day 4 (Q_{10}=1.87) was partially compensated as VO_{2R} decreased to 0.07 ml/h. Contrastingly, no such compensation was again observed in the metabolic rate of S_{10} individuals for which initial inhibition of CR lasted longer than in F mussels.

Thus, our results indicate the existence of two significant factors contributing to endogenous inter-individual differences in growth rate: i) the capacity to display an intense filtering activity which is functionally correlated with the gill-surface area and ii) the capacity to compensate the temperature effects on filtration and metabolic rate. The second trait seems to have insignificant contribution to the inter-individual size-differentiation in the mussels that were reared in warm environment (20 ºC), but explains a great proportion of inter-individual growth rate differences in cold environment (10 ºC). Moreover, since S_{10} individuals were inside the percentile 0-20 of the size-distribution of the population, it might be fairly concluded that at least 20% of the individuals in our mussel population possess ineffective molecular mechanisms of thermal acclimation.

Pernet et al. (2008) observed that intra-specific variation in physiological and biochemical adaptation to temperature correlates well with inter-individual variation in growth rate differences in the oyster *Crassotrea virginica.* They observed that selected lines of fast growing individuals were capable of reducing the unsaturation index of membrane lipids of gill-tissue more intensely than slow growers. In their study, a higher capacity for homeoviscous adaptation in the gill of fast growing oysters occurred concomitantly with i) lower standard metabolic rates and ii) higher clearance rates. Thus, they suggested that innate capability for thermal compensation of membrane fluidity could be a relevant factor explaining inter-individual growth rate differences. In conclusion, the two physiological processes underpinning inter-individual differences in growth potential observed in this study (i.e. the ability to display high clearance rates and the capacity for thermal compensation) point towards the gill as the key organ in determining growth rate variability, as previously suggested by Tamayo et al. (2011) and Prieto et al. (2018).

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Chapter 4

Molecular basis determining fast growing in the mussel *Mytilus galloprovincialis*

Abstract

Endogenously determined inter-individual differences in growth rate of bivalve molluscs have been widely analyzed at different organizational levels. Most studies have focused on the characterization of the physiological differences between fast (F) and slow (S) growing individuals. Although several genes have been described to be up regulated on fast growing individuals, the molecular basis underlying the mechanisms at the origin of growth variation is still poorly understood.

In the present study, we reared mussel spat of the species *Mytilus galloprovincialis* under diets below the pseudofaeces threshold (BP) and above the pseudofaeces threshold (AP). After 3 months, F and S mussels on each condition were selected, so that 4 experimental groups were obtained: F_{BP}, S_{BP}, F_{AP} and S_{AP}. We hypothesized that nurturing conditions during their growing period would modify the molecular basis of growth rate differences. However, results of feeding experiments showed that F mussels displayed higher clearance and ingestion rates and higher efficiencies of food selection prior to ingestion, as well as higher gill surface areas, irrespective of the rearing nutritional environment.

To decipher molecular mechanism at the origin of growth variation, gills of the 4 mussel groups were dissected, and used for transcriptome analysis with a custom Agilent single channel microarray. Gene expression analysis revealed i) a low number (12) of genes differentially expressed associated to *maintenance condition* differences and ii) 117 genes differentially expressed when comparing fast and slow growing mussels (F_{BP} + F_{AP} vs. S_{BP} + S_{AP}). We further investigated this comparison: GO terms and KEGG pathway association of the differentially expressed genes allowed us to analyze the functions involved on the differentially expressed encoding. Transcriptomic differences between F and S mussels were mainly based on the up-regulation of response to stimulus, growth and cell activity Biological Process GO terms. Regarding

the KEGG terms, carbohydrate metabolism and Krebs cycle were found to be up-regulated in F mussels whereas biosynthetic processes were up-regulated in S mussels.

Among the differentially expressed genes that are annotated, the following ones were found to be up regulated in F mussels: i) Mucin, related to mucus secretion, known to be crucial in food acquisition and pre-ingestive selection processes in bivalves, ii) genes related to growth such as Myostatin or Insulin-like growth factor, iii) genes involved in feeding activity, such as Fibrocystin or Dynein and iv) genes involved in the energetic metabolism; Citrate synthase. S mussels mainly over-expressed genes related to immune system and defence (Leucine-rich repeat-containing protein, Metalloendopeptidase, Small heat shock protein 24, Multidrug resistance,…).

The present results suggest that differences in feeding activity and in the allocation of metabolic energy between growth groups could account for the differences in growth rate in spat of *Mytilus galloprovincialis*. In accordance with their higher feeding rates and growth, fast growing mussels were found to mainly over-express genes involved in the development and maintenance of such activities, however, slow growing mussels needed to expend energy in immune and defence processes to ensure survival at the expense of growth rate.

Introduction

Inter-individual growth variability of bivalve molluscs has been reported to be endogenously determined (Brown, 1988; Dickie et al. 1984; Mallet and Haley, 1983; Pace et al. 2006; Tamayo et al. 2011). Studies comparing the physiological behaviour between fast and slow growing individuals have greatly contributed, in the last two decades, to the understanding of the physiological basis of differential growth. Main conclusions were that differences in growth rates resulted from differences in i) the capacity to acquire and absorb food, ii) the efficiency of energy conversion processes and/or iii) the allocation of energy to growth and maintenance (Koehn and Sumway 1982; Toro and Vergara, 1998; Bayne et al. 1999a, 1999b; Tamayo et al. 2014; Fernandez Reiriz et al. 2016).

The multi-locus heterozigosity hypothesis formulated by Sighn and Zouros (1978) stablished the existence of a positive correlation between the degrees of heterozygosity and growth rate. Aneuploidy was also demonstrated to play a role in

interindividual differences in growth rate in bivalves: significantly higher values of aneuploidy were observed in slow growing specimens of oysters (*Crassostrea gigas)* and more recently in the clam *Ruditapes decussatus* with a high negative correlation observed between growth rate and aneuploidy percentage (Leitao et al. 2001; Teixeira Da Sousa et al. 2011).

In recent years, with the aim of deciphering the underpinned mechanisms, high throughput gene expression analyses using next generation sequencing (NGS) or microarrays have allowed identifying genes involved in growth processes (Gracey et al. 2008; Lockwood et al. 2010; Susarellu et al. 2010; Devos et al. 2015; Suarez Ulloa et al. 2015; Xu et al. 2016) . For instance, Zhang et al. (2012) reported that collagen and laminins, (extracellular matrix proteins from connective tissue) and fibronectins are involved in the formation of the shell in the oyster *Crassostrea gigas*. Bassim et al. (2014) analyzing the gene expression of the mussel *Mytilus edulis* during early development (from egg to post-larvae) identified a set of genes related with growth processes in early development (*e.g.* GATAD1, PIP5K1A and ATRX) and highlighted (Bassim et al. 2015) 29 gene markers related to growth and mortality of bivalve larvae.

Very few studies have attempted to specifically analyze the differential gene expression between fast and slow growing specimens of bivalves. Using different crosses between inbred lines of *Crassostrea gigas*, Meyer and Manahan (2010) found significant differences between fast and slow growing larval families in the transcript abundance of ribosomal proteins as well as in the rates of expression of genes encoding for the small cardioactive peptide precursor (ScPB), involved in feeding regulation, and in several proteins involved in the energy metabolism. Some of them were electron transport components encoding genes (ND4L and ND1), ATP-synthase 8, and two coiled-coil- helix-coiled-coil-helix domains (CHCHD2 and CHCHD). More recently, De la Peña et al. (2016) reported the existence of significant differences in the rate of expression of ferritins (*Apfer1)* between fast and slow growing individuals of *Argopecten purpuratus* at different developmental stage (5 stages from embryos to juveniles). Wilson et al. (2016) produced an inbred fast growth line (F) of *Mya arenaria* clams and analyzed the gene expression to test the hypothesis that specific growth related genes will be up-regulated in F individuals. They established a positive correlation between some metabolic genes (fatty acid synthase and ATPase) with fast growth. They also found some up regulated genes involved in structural remodelling in

fast growing phenotype in agreement with previous studies pointing towards protein turnover as the main determinant processes for growth heterosis. Finally, Saavedra et al. (2017) concluded that genes controlling growth processes in model organism (GCGC) displayed a minor role in determining F and S in *Ruditapes decussatus* stocks. However, they found that the insulin-mediated processes had an essential role in interindividual differences in growth rate.

Although the available genetic information is increasing (Saavedra and Bachere, 2006; Tanguy et al. 2008; Astorga et al. 2014), the knowledge regarding molecular and genetic inter-individual differences in growth potential of bivalves remains at low standards. Large-scale sequencing projects (e.g. NGS) have produced broad amounts of sequences in databases, but most of them without an assigned function or similarity. Therefore, it is becoming evident that the combination of transcriptomic with other organizational levels response analyses is necessary to understand the roles of specific genes in the functional responses at the whole organism level (Bassim et al. 2014).

In the present study, we have analyzed the gene expression in gill tissue of mussels (*Mytilus galloprovincialis*) specimens that were selected as fast and slow growers under distinct nutritional environments. These mussels were used for recording physiological components of the Scope for Growth in order to assess the influence of rearing conditions on the parameters of physiological behaviour responsible for faster growth (Prieto et al. in preparation). Irrespective of feeding conditions during rearing, faster growers exhibited higher Scope for Growth values that mainly resulted from their increased capacity to acquire food. Indeed, fast growers displayed higher clearance rates and, consistently, they were found to have significantly higher gill-surface area per mass unit. Furthermore, these differences between fast and slow growing individuals were also observed when mussels are reared in environments submitted to air-exposure (Prieto et al. 2018 – chapter 2).

Thus, the gill is a candidate organ to play a major role in determining the inter-individual growth rate differences in the mussel *Mytilus galloprovincialis.* Accordingly, in the present study we have selected the gill tissue as the target organ to compare gene expression in these groups of fast and slow growing mussels. Aims of this study were to search for candidate genes for recorded differences in physiological behaviour and ultimately growth in order to ascertain biological processes accounting for such differences at the molecular level. Additionally, the effect of the rearing nutritional

condition was also considered as a possible modulator of molecular processes underlying inter-individual differences in growth rate. Specifically, emphasis was laid upon linking physiological (Prieto et al. in preparation- chapter 1) and transcriptomic results (present study) in order to achieve a more holistic understanding of the organism behaviour in different growth scenarios.

Material and Methods

Selection of mussels

Some 400 mussels (*Mytilus galloprovincialis*) of approximately 10 mm shell length (~150 mg live weight) were collected in a rocky shore in Antzoras (Bizcay, North Spain) in February 2014. Each of two groups of 200 mussels were alternatively fed with high quality diet (organic content = 80%) and a low quality diet (organic content = 30%) dosed at particle volume concentrations of 1.0-1.5 mm^3/L (below the pseudofaeces threshold (BP)) and 3.0-3.5 mm^3/L (above the pseudofaeces threshold (AP)), respectively. Shell-length was measured with a 0.05 accuracy caliber and and live-weight was determined using a 0.01 mg accuracy balance. After three months of maintenance under these feeding conditions fast (F) and slow (S) growing mussels were selected by choosing small and large specimens (percentiles $P_{12.5}$ and $P_{87.5}$ in the size distribution) to constitute 4 groups to be used in physiological experiments (chapter 1): i) fast growing mussels fed below the pseudofaeces threshold (F_{BP}), ii) slow growing mussels fed below the pseudofaeces threshold (F_{BP}), iii) fast growing mussels fed above the pseudofaeces threshold (F_{AP}), and iv) slow growing mussels fed above the pseudofaeces threshold (F_{AP}). The growth rates of the mussels were calculated as GR= the increase in shell-length or live-weigth/elapsed time (days). After physiological experiments were completed, the gills of the mussels were dissected and processed for gill surface area determination (chapter 1) and RNA extraction.

RNA extraction

Gill samples were stored immersed in RNA later at -80 ºC until the RNA was extracted with Ribopure system (Ambion kit). The analysis of the quality and integrity of RNA was checked with *Fragment Analyzer™* Automated CE System equipment from advanced analytical with *DNF-471 Standard Sensitivity RNA Analysis kit*, (15 nt) and Fragment Analyzer™ 1.1.0.11 software version. RNA quality was checked using PROSize 2.0.

Microarray design and hybridization

We used a SurePrint G3 Custom microarray (8x60k) from Agilent to analyse the transcriptome of the gill samples Microarray probes were designed using Agilent eArray platform, using *Mytilus galloprovincialis* sequences downloaded from NCBI in February of 2015. Sequences with the best blastx hit (e.value <$10e^{-10}$) to unique proteins against non-redundant database were selected. Three non identical probes were designed for each sequence. Housekeeping genes (those usually used in *Mytilus* qPCR analisis) were added as positive controls, alongside with default Agilent negative controls. The remaining spots in the array were filled with sequences of the genus *Mytilus* representing unique proteins (were *Mytilus galloprovincialis* ortolog was missing). Two probes of the unannotated sequence (one in each reading direction) were included in the array. Thus, the array was based on 17,491 unanotated and 7,806 annotated proteins. Hybridization was performed in 4 pools of 5 individuals per experimental group. Samples were quantified in the spectrophotometer UV/VIS Nanodrop 1000 (Thermo Fisher).

Marking protocol

We used "One-Color Microarray-Based Exon Analysis" marking protocol from Agilent. Samples were marked using "Low Input Quick Amp WT Labeling kit, One-Color" (p/n 5190-2943) kit. 100 ng of RNA were used for the marking reaction. Marked samples were quantified with Nanodrop ND-1000 to determine the efficiency of the specific activity of the fluorochromes.

Hybridization

Samples were manually hybridized with *SureHyb hybridization chambers* (Agilent technologies). Hybridization was done in the oven of Agilent technologies following the Agilent protocol. Characteristics: 600 ng of marked cRNA; 40 µl volume; 65 ºC temperature; 20 hours duration at 10 rpm in the hybridization.

Scanning

The scanning was carried out on the *DNA microarrays G2565CA* scanner with ozone –barrier slide covers with the *Scan control* Software version 8.5.1., using the default protocol *AgilentG3_GX_1Color.* The Scanning resolution was 3 µm and green channel, and the size of the resulting Tiff image was 20 bit.

Feature extraction

We used Agilent Feature Extraction Software (ver. 10.7.3.1) (Agilent Technologies) to process the microarray images and to quantify the fluorescence of the probes. The quality of all arrays was checked using the 9 QC-metric parameters generated in the feature extraction. Following this procedure, the fluorescence processed signal (generated by the feature extraction) was obtained.

Data treatment

Data treatment was carried out in R (v. 3.3.2.) using the Limma package (v. 3.30.13) from Bioconductor (Ritchie et al. 2015). Probes were pre-filtered using *gIsPosAndSignif* tag; a Boolean value indicating if the signal of the probe exceeds the background signal. Probes with a non-significant signal in all the samples of at least one experimental group (n=4) were removed. Background was corrected using *normexp* method and normalization between arrays was performed using *quantile* method, as described in Smyth et al. (2002). Fold-change and standard error were estimated by fitting the data to a linear model and an empirical Bayes (*eBayes)* smoothing was applied to the standard errors. The final gene expression value was the average of the non identical probes corresponding to each sequence. Differential expression quantification was based on a logarithmic scale (logFC), the adjusted p-value or False Discovery rate (Benjamin – Hochberg method) representing the statistical significance of the observed changes. Probes with FDR<0.05 were considered differentially expressed, as suggested in (Cheng and Pounds, 2007). A Hierarchical clustering (HCL) analysis was performed using Dendextend package (v.1.5.2.) to analyze similarity between samples.

Normalized hybridization values, as well as the raw data, were deposited in the gene expression omnibus (GEO) repository with the accession number GSE120975.

Annotation and gene ontology

Microarray sequences were annotated using Annocript 1.3. against Swissprot and Uniref databases (v. march-2017). Gene Ontology (GO) for three levels (Cellular Component, Molecular Function and Biological Process) was analyzed for transcriptome data interpretation, although we focused our analysis mainly on Biological Process (Suarez-Ulloa et al. 2015). GO terms list was summarized using

REVIGO (Supek et al. 2011). Differentially expressed genes were also mapped to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database for pathway analysis (Kanehisa, 2002). Conserved protein domains were identified using Prosite (Sigrist et al. 2009) and NCBI conserved protein domain finder tools.

Results

Growth rates of the experimental mussel groups

The live-weight, shell-length and growth rates of the four experimental mussel groups are shown in Table 1.1. The live-weight of F individuals was 2.5 fold higher than that from S individuals, and the shell-length was 45% larger. Accordingly, live-weight and shell-length growth rate of F individuals was found to be approximately 3 times higher than that of S individuals in both maintenance conditions (Table 4.1).

Table 4.1. Shell-length growth rate (mm/day) and live weight growth rate (g/day) (mean values ± SD) of F_{BP}, S_{BP}, F_{AP} and S_{AP} mussel groups.

Mussel Group	Growth rate (mm/day)	Growth rate (g/day)
F_{BP}	0.146 ± 0.009	0.012 ± 0.002
S_{BP}	0.055 ± 0.015	0.004 ± 0.001
F_{AP}	0.144 ± 0.007	0.011 ± 0.001
S_{AP}	0.060 ± 0.013	0.004 ± 0.001

Quality and reproducibility of the data

The RNA quality was good in all samples. The yield and the Cyanine 3 specific activity were higher than 0.825 µg/reaction and 15 pmol/µg respectively in all marked samples. Hybridization with the array suited (or passed) in all cases the quality standards, evaluated with 9 QC metrics parameters. Only in 0.95% of the probes (568 probes out of 59,539) the signal had a lower expression value than the background on all samples. For our analysis, we used the probes that had a positive signal on all the samples in at least one experimental group. Mean expression values and standard deviations of the housekeeping genes of the array are shown in the additional file 1. The variability among samples was lower than 3% in most of the housekeeping Genes.

Transcript annotation

The functional annotation of the genes on the array carried out by Blastx against Swiss-Prot and Uniref databases had 38.8% significant matches (E value 10^{-5}): 10,001 out of 25,781 genes. 27.7% of the annotated genes were matched on *Crassotrea gigas*, 10.2% on *Homo sapiens*, 8.4% on *Mus musculus,* and 3.6% on *Lottia gigantea*. 3.5% of the matches were found on distinct species of the genus *Mytilus* (*M. galloprovincialis* 1.8%, *M. coruscus* 0.5%, *M. trosulus* 0.5%, *M. edulis* 0.3% and *M. tax* 0.4%). There were 15,333 (3,142 unique) assigned Go terms on the annotated genes: 4,970 (32.4%) were Biological Process terms, 4,335 (28.3%) Molecular function terms and 6,091 (39.3%) Cellular Component terms. The KEGG ontology had 1,526 (378 Unique) matches.

Sample Distribution

We performed a hierarchical clustering (HCL) with the whole transcriptomic data to analyze the similarity in gene expression pattern between all samples. Two clusters were obtained on the HCL analysis of the transcriptome (Figure 4.1). First cluster included mostly slow growing mussels: 6 out of 8 S mussel pools were grouped in this cluster, while most of F mussel pools 7 out of 8, were grouped in the second cluster. Not clear differentiation pattern according to maintenance diet (BP vs AP) was found.

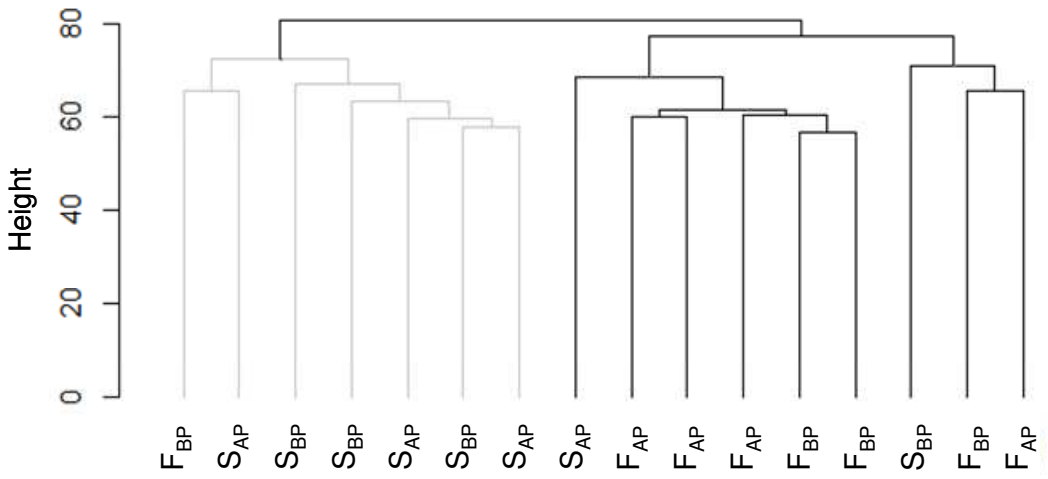

Figure 4.1. Hierarchical clustering (HCL) of gene expression of fast (F) and slow (S) growing mussels of BP (Below Pseudofaeces threshold) and AP (Above Pseudofaeces threshold) conditions. The two main clusters obtained by the HCL are marked in grey and black.

Identification of differentially expressed genes

We performed 6 comparisons (FDR 5%) to analyse the effect of *growth condition* and *maintenance condition* on the transcriptome of gill tissue (Table 4.2).

Table 4.2. Number and fraction (DEG/total genes in the microarray) of differentially expressed genes (adj.p.value <0.05) on the 6 comparisons between experimental groups to test the molecular effect of *growth condition* and *maintenance condition* factors.

Tested factor	Group Comparison	Up-regulated	Down-regulated	Fraction
Growth condition	F_{BP} vs S_{BP}	5	2	0.03
	F_{AP} vs S_{AP}	1	0	0.00
	F vs S	70	47	0.51
Maintenance condition	F_{BP} vs F_{AP}	1	2	0.01
	S_{BP} vs S_{AP}	2	1	0.01
	BP vs AP	7	5	0.05

No strong effect of diet quality on transcriptome profile was found since BP vs AP differences amounted to only 0.05% (12 genes; 7 up- and 5 down-regulated). Effects were still less important when these quality differences are analyzed by growth categories (0.01%). Regarding the *growth condition* factor, 117 genes (0.51%) were differentially expressed (70 up- and 47 down-regulated) between F and S mussels, although the number of DEG decreased when F vs. S comparisons were performed for each diet.

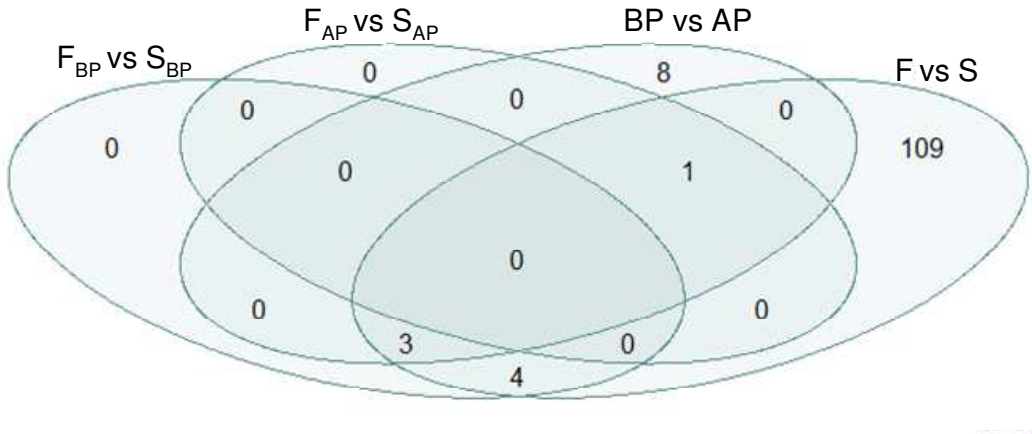

Figure 4.2. Venn diagram showing the redundancy of differentially expressed genes between the four selected comparisons of mussels (F_{BP} vs. S_{BP}; F_{AP} vs. S_{AP}; BP vs. AP; F vs. S).

Consequently, we searched for redundancy in DEG among the four comparisons (F_{BP} vs. S_{BP}; F_{AP} vs. S_{AP}; BP vs. AP and F vs. S) using a Venn diagram (Figure 4.2). All DEGs of the 1st and 2nd comparison were also found to be differentially expressed in the whole comparison of F vs S mussels. Conversely, regarding the comparison of the maintenance conditions (BP vs. AP), only 4 out of 12 genes were found commonly differentially expressed with F vs. S. No common differentially expressed genes were found for the 4 comparisons.

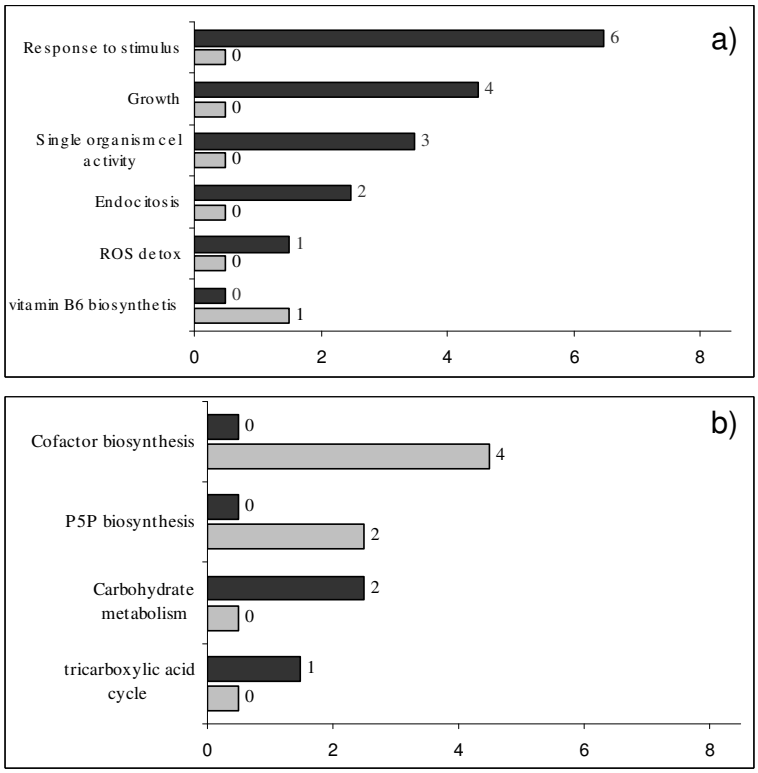

Figure 4.3. Graphical representation of the up (black) and down-regulated (grey) Biological Process GO terms (a) and KEGG pathway terms (b) in F mussels in comparison with S mussels. The length of the bars on Biological Process graph represents the number of different GO composing each group after REVIGO summarization. KEGG bars length represents the count of the repeteance of specific pathway.

Resource to GO term and KEGG pathway association of genes differentially expressed in the comparison between F and S mussels (F vs. S) was intended to achieve a functional interpretation of changes in the transcriptome that are assumed to encode for quantitative differences in growth. A comparison of the functional profiles for Biological Process and for the KEGG pathways of the selected four comparisons is shown in Figure 4.3. Main transcriptomic difference between F and S mussels was

accounted for by up-regulation of response to stimulus (37.5%), growth (20%) and cell activity (18.75%) processes. Regarding the KEGG pathway on S individuals, cofactor and pyridoxal-5-phosphate biosynthesis pathways were found to be up-regulated on S individuals and carbohydrate metabolism and tricarboxylic acid cycle pathways were found to be up regulated on F individuals. A list of annotated differentially expressed genes is shown in Table 4.3.

Table 4.3. List of differencially expressed genes in F versus S comparison. Gene name, E-value and description were obtained with Annocript by Blastx against Swissprot and Uniref databases. Log FC: Log2 fold change. P: adjusted p value.

Comparison	Regulation	Gene name	E-value	Description	Log FC	P
F vs S	Up	Q05049 / A0A1L8HCH0	$1.00E^{-16}/2.00E^{-15}$	Integumentary mucin C.1 (Fragment)	3.97	0.042
		Q80ZA4 / K1Q166	0.0/0.0	Fibrocystin-L	1.89	0.001
		-/K1Q7V2	$-/3.00E^{-10}$	DENN domain-containing protein 3	1.58	<0.001
		A0MSJ1 / K1PT11	$7.00E-15/5.00E^{-13}$	Collagen alpha-1(XXVII) chain B	1.37	0.034
		Q60754 / UPI00042A9BFF	$8.00E-08/4.00E^{-10}$	Macrophage receptor MARCO	1.33	0.044
		Q9WVT6/K1QCX8	$4.00E-18/2.00E^{-26}$	Carbonic anhydrase 14	1.30	0.003
		- / S4UD24	$-/8.ooE^{-09}$	Nitric oxide synthase	1.18	0.019
		Q5USW0 / T1WDY6	$6.00E^{-31}/1.00E^{-99}$	Growth/differentiation factor 8- Myostatin	1.13	0.001
		P21793/UPI0005C3B0DE	$2.00E^{-14}/9.00E^{-91}$	Decorin	1.02	0.009
		- / K1P9F1	$-/2.00E^{-18}$	Insulin-like growth factor-binding protein complex acid labile chain	0.95	0.035
		P37889 / K1QKY6	$1.00E^{-16}/2.00E^{-32}$	Fibulin-2	0.66	0.021
		Q8WXX0 / K1QK11	$4.00E^{-145}/0.0$	Dynein heavy chain 7 axonemal	0.66	0.023
		Q4R3F0/UPI0005C3C6F2	$8.00E^{-07}/0.0$	Protein tilB homolog	0.60	0.022
		P02469 / UPI00097509DE	$4.00E^{-176}/0.0$	Laminin subunit beta-1	0.59	0.038
		Q4S5X1 / A0A0L8GP61	$1.00E^{-94}/5.00E^{-103}$	Citrate synthase mitochondrial	0.56	0.007
	Down	- / A0A0A7AD04	$-/3.00E^{-25}$	CRP-I 9	-4.39	0.021
		Q922Q8 / J9Q3A8	$2.00E^{-45}/2.00E^{-54}$	Leucine-rich repeat-containing 59 (Fragment)	-3.38	0.016
		Q16820 / A0A194ALD8	$1.00E^{-17}/3.00E^{-123}$	Metalloendopeptidase	-3.35	0.021
		- / Q8MW54	$-/8.00E^{-24}$	Precollagen-P	-3.08	0.007
		O34245 / K5UM09	$1.00E^{-57}/5.00E^{-111}$	Anaerobic C4-dicarboxylate transporter	-2.98	0.034
		P06582 / G3GAE5	$3.00E^{-07}/5.00E^{-172}$	Small heat shock protein 24.1	-2.84	0.014
		Q8WPW2 / H9LHX0	$7.00E^{-81}/4.00E^{-97}$	Putative pyridoxine biosynthesis SNZERR (Fragment)	-2.51	0.016
		Q86IV5 / K1R157	$5.00E^{-40}/2.00E^{-118}$	Countin-1	-1.96	0.041
		Q9R1X5 / K1PW26	$2.00E^{-31}/2.00E^{-82}$	Multidrug resistance-associated protein 5	-1.81	0.021
		P56470 / A0A0C5Q4G0	$2.00E^{-53}/1.00E^{-147}$	Galectin	-0.94	0.035

Discussion

In the present study, we have analyzed the molecular differences between experimental mussel groups considering the physiological performance of each group in their maintenance conditions. The similitude of transcriptomic profiles (HCL results) and low number of differentially expressed genes between mussels reared at BP and AP conditions are in accordance with the fact that only slight physiological differences were found between these groups (chapter 1, Tables 1.3 and 1.4). Physiological differences

between F and S mussels were similar, i.e. *maintenance condition* did not exert significant effect on the physiological parameters of the mussels. This pattern was also observed in mussels reared under similar feeding conditions but submitted to a daily air exposure (Prieto et al. 2018 – chapter 2). This results indicate that whereas a switch in the feeding routine of the mussels (from continuous production of pseudofaeces in AP diets to increased CR in BP diet) involves minor changes in the transcriptome, the endogenously determined variability in the capacity to grow implies the differential expression of a significantly higher amounts of genes.

We have found 117 differentially expressed genes in gill tissues underlying the differences in growth rate between fast and slow growing specimens. The classification of these genes according to Biological Process GO terms indicated that the molecular differences mainly refer to response to stimulus, growth and cellular activity processes. Thus, the GO term findings supported the higher growth rates and activity levels of F individuals in comparison with S mussels. Not surprisingly, previous works on inter-individual growth rate differences in bivalves have also described similar GO terms as the main processes underlying growth differences: For instance, Wilson et al. (2016) described that 19% of GO terms of differentially expressed genes in fast growing *Mya arenaria* refer to cell structure, whereas 17% referred to signalling and growth, 12% to energy and nutrient metabolism and 10% to DNA/RNA and protein synthesis. Regarding the KEGG terms, energetic metabolism terms were referred to F individuals in good correspondence to their higher activity levels. Up-regulation of Cofactor and P5P biosynthesis pathway in S individuals seemed to involve differences in protein metabolism that could underlie differences in the *protein turnover* between growth groups, as described in previous studies (Hawkins et al. 1986, 1996). P5P biosynthesis pathway could either indicate a higher rate of anaerobic metabolism, which in bivalves is based on the utilization of aminoacids via opine dehydrogenases or asparte-succinate pathway (Hochochka and Somero 2002)

Most differentially expressed (DE) genes between present F and S individuals lack a clear association to GO terms because the studied model presents only few sequences annotated in the tools allowing to perform GO analyzes. Thus, in what follows emphasis has been laid upon the individual (rather than group) analysis of DE genes and their functions to decipher the molecular basis of growth rate differences.

Up-regulated genes in F mussels.

Up-regulation of Growth Differential Factor-8 and Insulin Like Growth Factor, both involved in growth regulation processes, in F mussels would appear meaningfully associated to the higher growth rates exhibited by this group. Growth differential factor-8, also known as myostatin, is a protein of the transforming growth factor-β superfamily. It is an important negative regulator of muscle growth in vertebrates, but the ubiquitous expression in bivalve tissues suggests that it could have alternative functions related with cell development (Saina and Technau, 2009; Nuñez Acuña and Gallardo-Éscarate, 2014; Morelos et al. 2015; Niu et al. 2015). Besides, differential growth traits have been found to be correlated with different polymorphism in myostatin gene (Wang et al. 2010). Our findings suggest that myostatin might have an important role in growth processes underlying differences between F and S individuals. Insulin like peptides, expressed on various tissues such as labial palps or mantle on bivalves are important growth regulators of soft tissues and shell (Taylor et al. 1996; Gricourt et al. 2003). The role of insulin like growth factors in inter-individual growth rate differences in bivalves have been recently suggested by Saavedra et al. (2017), who found the over expression of NOV-like protein in the gills of fast growing *Ruditapes decussatus*. Additionally, using Prosite tool on the highly differentially expressed genes (FC>8, FDR<0.01) we have found a possible epidermal growth factor-like (EGF) domain signature up-regulated in F individuals. Valenzuela-Miranda et al. (2015) also reported the over expression of EGF in the muscle tissue of F specimens of the abalone *Haliotis rufescens*. EGF binds to EGFR (epidermal growth factor receptor) resulting on cell proliferation and growth (Herbst RS, 2004). Although the knowledge of EGF role in bivalves is limited, its expression has been reported in various tissues (e.g. muscle and mantle) and it appears to induce cell proliferation and migration during wound healing (Sun et al. 2014) and to stimulate two glycolytic enzymes: phosphofructokinase (PFK) and pyruvate kinase (PK) (Canesi et al. 2000).

In addition to the growth-regulators supporting the higher growth rates, gills of F mussels were found to over express some genes involved in the structure and functionality of the connective tissue. Confirming present results, some of these genes have been previously reported to be differentially expressed between fast and slow growing individuals of different mollusc species in other studies. E.g. collagen, an essential component of the extracellular matrix, that has an important role on shell-

formation process (Zhang et al. 2012) and known to be involved in soft tissue growth and repair processes (Zhao et al. 2012), has been found to be up regulated in F individuals of the abalone *Haliotis rufescens* (Valenzuela-Miranda et al. 2015) and the clam *Ruditapes decussatus*, (Saavedra et al. 2017). As in the present study, Valenzuela –Miranda et al. (2015) also observed the over expression in F individuals of laminin, known to bind components such as collagen to the extracellular matrix. Additionally, gills of F individuals were found to over express other proteins known to interact with collagen: fibulins and decorins. Fibulins have an important role in development and biomineralization processes in association with both laminin and collagen (Timpl et al. 2003; Sleight et al. 2015), and decorin is a component of extracellular matrix playing an important role in fibrillogenesis (binding with collagen). Decorin is known also to interact with some growth factors, as epidermal growth factor receptor, up regulated in F individuals in the present study, and its binding with myostatin has been recently described to cause hypertrophy in human muscle cells (Kanzleiter et al. 2014). Decorin function has not been described in bivalves up to date; however, its up-regulation on F individuals in the present study is in accordance with the over-expression of two main interactional proteins: collagen and myostatin, and thus decorin could be involved on growth processes in bivalves.

Up-regulation of the above genes involved in growth regulation and structural components of the connective tissue of F individuals is consistent with their recorded higher growth rates and larger gills (chapter 1, Table 1.1 and Figure 1.7). Indeed, this set of transcriptomic features are consisting, at the molecular level, with higher rates of cell renewal supporting higher gill surface area and higher filtering activity.

It is also highly meaningful the finding of four up-regulated genes in F individuals related with feeding processes since the main physiological difference between fast and slow growing mussels was the enhanced capacity for energy acquisition of F specimens, promoted by their higher clearance rates. The importance of gill size and turn-over rate of branchial cells in these processes has just been discussed in association with the over-expression of structural components but complementarily F mussels over-express also proteins more properly related to the muco-ciliary function. For example, F mussels over expressed mucin, a key component in mucus secretion that has been related with many different activities such as movement, immunity, nutrition or activity. The complexity of the proteomic composition and function of mucus in

different organs of the oyster *Crassostrea virginica* has been recently described (Espinosa et al 2016). In the gills, mucus has an essential role in defence against pathogens and particle processing, mainly in food acquisition and pre-ingestive selection processes (Beninger and St-Jean 1997; Urrutia et al. 2001; Espinosa et al. 2014). Filtered particles are retained in the mucus of the gills and transported by the cilia to be ingested or rejected within a mucus string in pseudofaeces form. The major mucus production in F individuals would be in concordance with their higher clearance rates and higher pre-ingestive selection efficiencies, both physiological parameters greatly contributing to inter-individual differences in growth rate of these mussels. Fibrocystins are involved in tubulogenesis processes and seem to have an essential role in the correct functioning and/or structure of kidney primary cilia (Ward et al. 2003). Although the function of the fibrocystin on the cilia remains unclear, the up-regulation found in present study in gills of F mussels that have significantly higher clearance rates, suggests that fibrocystin could have an important role on filtering processes. Additionally, the findings of dynein and tilb homolog protein genes, whose products are involved in ciliary motility is also in good correspondence with the higher pumping rate of water reported for these mussels. Dynein are motor proteins moving along microtubules, capable of converting ATP hydrolysis into mechanical work which is responsible of the motility by producing the force for beating (Gibbons et al. 1965; Horani et al. 2013) and Tilb is involved on dynein arm assembly and also has an essential role in ciliary motility processes (Kavlie et al. 2010). Recently Espinosa et al (2016) characterized dynein protein in the mucus of the gills of the eastern oyster *Crassostrea virginica,* and interestingly, Valenzuela-Miranda et al (2015) found dynein overexpression in F specimens in Californian red abalone (*Haliotis rufescens*).

Processes involved in metabolic energy supply and ATP turnover are specially relevant as regards growth rate of bivalves, and thus, the finding that 2 genes related with energy metabolism were up-regulated in F individuals is highly meaningful. Previous approaches to the characterization of molecular differences between fast and slow growing individuals of different species had emphasized the importance of differential aspects of the energetic metabolism between growth lines. For instance, Meyer and Manahan (2010) found ATP-synthase and two different NADH dehydrogenase subunits up regulated in F individuals of the oyster *C.gigas,* Wilson et al. (2016) found fatty acid synthase like-1 and fatty acid synthase like-2 genes up-

regulated in fast growing individuals of *M.arenaria* and Saavedra et al. (2017) found NADH subunit upregulated in F individuals of the clam *Ruditapes decussatus.* In the present study, we found citrate synthase (CS) and carbonic anhydrase up-regulated in F individuals indicating an enhanced aerobic energy metabolism as previously described in other studies, and in accordance with the higher activity level of F individuals. Citrate synthase is an specific marker of aerobic metabolism and has been shown to correlate with respiration rates in facultative anaerobes such as intertidal invertebrates (Dahlhoff et al. 2002), and considered as an indicator of the general physiological status of the organism (Garcia Esquivel et al. 2001, 2002; Pernet et al. 2012; Guévélou et al. 2013). Higher citrate synthase expression in our F mussels might thus be indicative of increased energy requirements of gill tissue to sustain higher filtering activity. Carbonic anhydrase enzyme family maintains the acid balance and and favours the exchange of respiratory gases (Breton, 2001). The over-expression of ~~both~~ citrate synthase and carbonic anhydrase are indicative of an enhanced aerobic energy metabolism.

Finally, DENN Domain Containing 3, also found to be up-regulated in F individuals, is involved in the conversion of inactive GDP-bound to GTP form and vesicle mediated transport pathways (Marat et al. 2011). We have not found evidences of DENN domain containing protein function in bivalves.

Up-regulated genes in S mussels.

Gills and digestive tract are known to be a target organ of pathogens or pollutants because they act as the first barrier against them (Regoli and Principato 1995; Rajalakshmi and Mohandas, 2005; Allam and Espinosa, 2016). Higher clearance rates recorded for fast growing mussels in present experiments imply that F-mussels pump largest volumes of water per time unit than S mussels to the pallial cavity and filtering structures. Accordingly, F mussels would be expected, *a priori* to be more riskily exposed to pathogens and, consequently, to develop stronger immune and defence responses than slow growing individuals. However, and contrary to our expectations the gills of the slow growing mussels over express many genes involved in immune and defence responses, such as HSP24, leucine rich repeat proteins, metalloendopeptidase and galectin. Over-expression of heat shock proteins has been commonly found in organisms maintained under temperature stress (Hofman and Somero 1996; Somero 2012; Lockwood et al. 2013), salinity stress (Zhao et al. 2012) metal exposure (Zhang et

al. 2012) and/or bacterial exposure (Genard et al. 2013). Besides, Zhang et al. 2012 found an over-expression of HSP genes in the oyster *Crassostrea gigas* under various stress conditions (Air exposure, thermal stress, salinity stress and metal exposure) and concluded that HSP induction could be a common defence against all stresses in *C. gigas*. Leucine rich repeat proteins has been described to be involved in the immunity of invertebrates (Wang et al. 2016) and metalloendopeptidase, seems to be key component of the response against bacterial infections (Miyoshi et al. 2000). Galectin is a lectin family protein that has been described to play a significant function in the immune response of oysters against the protozoa *Perkinsus marinus* (Nikapitiya et al. 2014). In good correspondence with present study, Saavedra et al. (2017) also found differences between fast and slow growing individuals of the clam *Ruditapes decussatus* in the immune and defence processes of digestive gland and gills. S individuals over expressed genes involved in immune and defense processes such as defensin and tumor necrosis factor member 11, whereas F individuals were found to over express different genes, such as sialic acid-binding lectin and hydramacin-1. They conclude that the observed high differences in the expression of immune and defence genes could reflect a differential fitness among individuals, promoting faster growth rates in those individuals able to fight more efficiently against diseases. Although in the present study most of the immune and defence genes were found to be over expressed in S mussels, F individuals were found to up-regulate MARCO, involved in antimicrobial immune system in mammals, whose function in bivalves remains unknown (Moreira et al. 2015).

Up-regulation of genes involved in immune and stress responses found in S mussels strongly suggests a greater prevalence of pathogens/diseases or a higher susceptibility to the pathogens. As suggested by Genard et al. (2013) when analyzing the physiological response of *C.gigas* larvae submitted to bacterial infection, extra investments in supporting defence mechanisms might drain energy resources from normal processes in healthy organisms, resulting in reduced feeding and growth performances. Strong up-regulation of countin-1 (FC≈4), a cell-counting factor that limits the maximum size of the multicellular structure by the down-regulation of the cell adhesion mediator gp24, is indicative of developmental processes inhibition at the molecular level in S individuals. Symptoms of impairment in the respiratory function of the gill affecting aerobic ATP production are also evident in S mussels: Evidences of increased use of anaerobic metabolic pathways include strong up-regulation of

anaerobic C4-dicarboxilate transporter (FC ≈ 8) as well as the increased biosynthesis of pyridoxal-5 –phosphate. Similarly, Saavedra et al. (2017) have reported up-regulation in the digestive gland of S clams of enzymes very likely involved in anaerobic metabolism (e.g. malate dehydrogenase and glicerol-3-phosphate dehydrogenase)

Conclusions and prospects

The present results show the existence of substantial differences in the transcriptome of the gills of F and S individuals. The gill of the fast growing mussels over expressed genes that are involved in the development and maintenance of cellular activity which suggest that F individuals are well equipped to maintain higher filtering activities that enable them to maximize food acquisition and sustain fast growth rates. On the contrary, slow growing mussels over express genes involves in immune processes, cellular stress and anaerobic metabolism suggesting that S individuals might need to spend higher rates of metabolic energy to fight against exogenous stressors, thus, reducing the scope for growth of the organism. Thus, immunity response seems to be crucial in determining the growth rate on *Mytilus galloprovicincialis* mussel spats.

We have reported here a list of differentially expressed genes between fast and slow growing phenotypes of the mussel *Mytilus galloprovincialis* that could be useful as molecular markers in breeding programmes in order to select families of high growth rates.

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Additional files

Table 4.A1. Expression of housekeeping genes: mean values (± SD) of the experimental mussel groups.

	F_{BP}	S_{BP}	F_{AP}	S_{AP}
Elongation factor 1-alpha	15.64 ± 0.08	15.61 ± 0.09	15.66 ± 0.09	15.68 ± 0.14
Tubulin alpha-1 chain	14.92 ± 0.12	14.92 ± 0.12	14.80 ± 0.06	14.87 ± 0.20
Ribosomal protein S15 (Fragment)	15.23 ± 0.11	15.11 ± 0.08	15.12 ± 0.14	15.17 ± 0.12
40S ribosomal protein S4 (Fragment)	16.59 ± 0.08	16.50 ± 0.12	16.59 ± 0.06	16.54 ± 0.09
Collagen protein (Fragment)	6.64 ± 0.11	6.61 ± 0.27	6.41 ± 0.02	6.40 ± 0.12
-	6.41 ± 0.38	6.22 ± 0.10	6.23 ± 0.10	6.11 ± 0.09
Actin adductor muscle	16.88 ± 0.16	16.90 ± 0.08	16.85 ± 0.12	16.90 ± 0.18
60S acidic ribosomal protein P0	16.76 ± 0.12	16.69 ± 0.08	16.73 ± 0.06	16.76 ± 0.04
-	6.25 ± 0.10	6.24 ± 0.05	6.38 ± 0.15	6.28 ± 0.04
-	11.72 ± 0.05	11.65 ± 0.06	11.71 ± 0.11	11.56 ± 0.05
Precollagen-NG	9.16 ± 0.39	7.99 ± 0.36	9.47 ± 0.55	8.02 ± 0.18
E3 ubiquitin-protein ligase UBR2	9.74 ± 0.07	9.62 ± 0.10	9.74 ± 0.09	9.58 ± 0.11
Ubiquitin carboxyl-terminal hydrolase isozyme L5	9.27 ± 0.15	9.19 ± 0.16	9.26 ± 0.20	9.32 ± 0.13
Ubiquitin carboxyl-terminal hydrolase 40	7.50 ± 0.14	7.41 ± 0.12	7.54 ± 0.08	7.46 ± 0.14
E3 ubiquitin-protein ligase RNF8	7.51 ± 0.08	7.54 ± 0.11	7.54 ± 0.19	7.39 ± 0.09
Ubiquitin-conjugating enzyme E2 L3	9.30 ± 0.20	9.53 ± 0.03	9.46 ± 0.13	9.49 ± 0.08
Uncharacterized protein	6.22 ± 0.04	6.39 ± 0.33	6.28 ± 0.23	6.30 ± 0.19
PREDICTED CTTNBP2 N-terminal-like protein partial	7.64 ± 0.06	7.59 ± 0.07	7.62 ± 0.01	7.54 ± 0.06
Collagen type IV alpha-3-binding protein	10.87 ± 0.10	10.78 ± 0.06	10.66 ± 0.09	10.74 ± 0.08
E3 ubiquitin-protein ligase TRIM71	7.51 ± 0.22	7.33 ± 0.11	7.48 ± 0.13	7.37 ± 0.12

Conclusiones generales

1.- La serie de experimentos realizados en esta tesis demuestran la existencia de un elevado grado de diferencias inter-individuales en la tasa de crecimiento en semillas del mejillón *Mytilus galloprovincialis* mantenidas bajo condiciones estables en el laboratorio. Las condiciones de estabulación de los mejillones en el laboratorio garantizaban que el acceso al alimento y al oxígeno era idéntico para todos los individuos. Pues bien, en todos los experimentos realizados, en los que se los mejillones fueron mantenidos bajo diferentes condiciones experimentales, se ha verificado que los individuos que presentaron las tasas de crecimiento más elevadas (designados F: *fast*) doblaban o triplicaban en tasa de crecimiento a aquellos que presentaban los valores más reducidos de tasa de crecimiento (designados S: *slow*). Esta notable diferencia inter-individual en la capacidad para crecer se ha obtenido en mejillones mantenidos tanto bajo condiciones de alimentación óptima (alimentación e inmersión continuas) como sometidos a distintos grados de restricción nutricional (discontinuidad en la inmersión o alimentación restringida a un único día por semana) tanto a temperaturas templadas (20 ºC) como frías (10 ºC). La constancia en la aparición de diferencias inter-individuales en tasa de crecimiento, con la consiguiente diferenciación en tamaño, en mejillones sometidos a condiciones estables y homogéneas, es decir idénticas para todos los individuos, demuestra la existencia de diferencias interindividuales de carácter endógeno en la capacidad de crecimiento en el mejillón *Mytilus galloprovincialis*.

2.- Las diferencias interindividuales en tasa de crecimiento empíricamente registradas en mejillones sometidos a condiciones idénticas en el laboratorio se corresponden con diferencias interindividuales significativas en los parámetros fisiológicos que determinan el balance energético individual. Los mejillones segregados por su crecimiento rápido (F) bajo las distintas condiciones de mantenimiento que se han ensayado en la presente tesis (distintos niveles de restricción nutricional y calidad de la dieta y distintas temperaturas de aclimatación) muestran balances energéticos significativamente superiores a los de sus congéneres de crecimiento lento (S) bajo el conjunto de condiciones experimentales testadas. La serie de experimentos desarrollados con mejillones F y S seleccionados bajo diferentes regímenes nutricionales muestran que el balance energético es superior y menos dependientes de

las características de la dieta experimental en los individuos de crecimiento rápido (F) que en los individuos de crecimiento lento (S).

3.- El conjunto de experimentos de la tesis han tenido como objetivo confirmar o rechazar la hipótesis de que las características fisiológicas que generan diferencias entre individuos de crecimiento rápido (F) y lento (S) pueden variar en función de las condiciones ambientales bajo las cuales se desarrolla el crecimiento de los mejillones. Las evidencias obtenidas en los distintos experimentos muestran que, efectivamente, las variables ambientales ensayadas (disponibilidad de alimento y temperatura del agua) pueden alterar las bases fisiológicas sobre las que se sustenta la existencia del crecimiento diferencial entre individuos. Esta constatación permite indicar que las diferencias en tasa de crecimiento no obedecen a diferencias en un único proceso fisiológico, sino a múltiples rasgos fisiológicos cuya contribución al establecimiento de diferencias inter-individuales en tasa de crecimiento depende de las características del medio ambiente en el que se desarrolla el crecimiento.

4.- En condiciones de alimentación continua (capítulo 1 y tratamiento 1 del capítulo 2), tanto con dietas de calidad baja (materia orgánica diluida con sedimento en concentraciones situadas por encima del umbral de formación de pseudoheces) como de calidad alta (porcentaje superior al 80 % de fitoplancton y dosificado por debajo del umbral de formación de pseudoheces), y tanto si son sometidos a condiciones de inmersión continua como discontinua (es decir, con periodos de exposición al aire como los impuestos por el ciclo mareal en las poblaciones intermareales) las diferencias fisiológicas fundamentales que subyacen en la segregación de individuos de alta y baja tasa de crecimiento fueron las siguientes: a) Los individuos F presentan una significativamente mayor capacidad para la adquisición de alimento, es decir, desarrollan tasas de aclaramiento por unidad de peso (una vez estandarizada para un peso común) significativamente superior a la que presentan los individuos S. b) Los individuos F presentan valores significativamente superiores de eficiencia de selección pre-ingestiva que los individuos S, lo que significa que el aparato filtrador de los F se encuentra mejor dotado para seleccionar e ingerir preferencialmente la materia orgánica disponible en el alimento. c) A pesar de presentar mayores tasas de ingestión, la eficiencia de absorción de alimento en los F no resulta mermada, o al menos no tanto como para impedir que su tasa de absorción no sea también significativamente superior que la de sus congéneres S. d) A pesar de absorber más alimento, los individuos F no

presentan tasas metabólicas por unidad de peso (una vez estandarizada para un peso común) significativamente superiores tanto en lo que se refiere al metabolismo de rutina como al metabolismo estándar.

En contraste, con lo anteriormente expuesto, en condiciones de restricción nutricional severa (tratamiento 2 del capítulo 2), condición que se simuló restringiendo el régimen de alimentación a un único día por semana, la diferencia fundamental entre individuos de crecimiento rápido y lento consistió en que los individuos F presentaron una tasa metabólica estándar significativamente menor que la de los S, resultado que sugiere que el relativamente superior crecimiento de los F se debería a su mayor capacidad para ahorrar energía durante los largos períodos de ayuno a que fueron sometidos en esas condiciones de severa restricción alimentaria.

Estos resultados nos permitieron definir lo que se podría interpretar como dos fenotipos básicos de individuos de alta tasa de crecimiento en el mejillón: i) aquellos que presentan una innata mayor capacidad para adquirir y procesar el alimento "*Fast feeders"* y ii) los que presentan una mayor capacidad para reducir los costes metabólicos basales, lo que les permite gastar menos energía en los periodos de ausencia de alimento "*Energy savers"*. En el capítulo correspondiente (capítulo 2) se ha discutido las implicaciones de dicha dualidad para el éxito o fitness de las poblaciones de mejillones que, en el medio natural, experimentan periodos alternos de abundancia y escasez de alimento. Los resultados obtenidos con los mejillones del tratamiento 2 del capítulo 2 demostraron que los individuos que más crecieron en las condiciones de severa restricción nutricional (caracterizados por presentar menores tasas metabólicas estándar: *energy savers*) sometidos a condiciones de alimentación continua acabaron desarrollando tasas de aclaramiento significativamente superiores a los S tras un período de aclimatación de 11 o 12 días. Aunque se requerirían nuevos experimentos para confirmar los resultados obtenidos en este capítulo, la conclusión provisional que se obtiene de la serie de experimentos presentados en el capítulo 2 es la de que los mejillones de crecimiento rápido tendrían la capacidad de adaptarse a las circunstancias ambientales comportándose como *fast feeders* bajo condiciones de bonanza nutricional y como *energy savers* bajo condiciones de severa restricción nutricional.

5- Un aspecto interesante de las características fisiológicas de los individuos de crecimiento rápido es que no solo poseen una mayor capacidad para desarrollar tasas de aclaramiento elevadas, sino que además presentan eficiencias de selección pre-ingestiva

significativamente superiores que los mejillones S, tanto si la segregación entre F y S tiene lugar con dietas bajo las que no se producen pseudoheces (dietas de alto contenido orgánico y moderada concentración total de partículas) como si son criados con dietas que provocan la necesidad de recurrir de manera continua a la producción de pseudoheces (dietas de bajo contenido orgánico y alta concentración de partículas). La concurrencia de mayores tasas de aclaramiento y mayores eficiencias de selección pre-ingestiva incluso en los individuos de crecimiento rápido (F) segregados en condiciones de alimentación por debajo del umbral de pseudoheces sugiere que ambos rasgos (la capacidad para realizar una mejor selección pre-ingestiva y el de desarrollar mayores tasas de aclaramiento) estarían funcionalmente ligados.

6- Hemos hallado evidencias que indican que las diferencias endógenas en la eficiencia de los mecanismos de compensación térmica del metabolismo y la tasa de aclaramiento a las temperaturas bajas (10 ºC) también contribuye a las diferencias interindividuales en tasa de crecimiento en el mejillón *Mytilus galloprovincialis*. Tanto los individuos F como S segregados a temperaturas cálidas presentaron respuestas compensatorias similares, en términos de tasa de aclaramiento y metabolismo de rutina, cuando eran sometidos a cambios agudos en la temperatura de exposición. Sin embargo, en los mejillones segregados a bajas temperaturas, únicamente los individuos F fueron capaces de desarrollar mecanismos de compensación térmica cuando eran sometidos a incrementos agudos en la temperatura de exposición. Además, el consumo de oxígeno estándar y de rutina de los mejillones de crecimiento lento (S) en temperaturas bajas (10 ºC) resultó ser inferior, no sólo a los de sus congéneres F, sino también al de los mejillones F y S criados a 20 ºC tras permanecer dos semanas aclimatándose a 10 ºC. Este resultado indica que los mejillones que se seleccionaron por su crecimiento lento a temperaturas bajas carecen de mecanismos de aclimatación térmica o respuestas de compensación crónica.

7- Los individuos de crecimiento rápido (F) han mostrado poseer una superficie branquial (superficie de área branquial por unidad de peso) significativamente mayor que los individuos de crecimiento lento (S). La existencia de dicha diferencia se ha verificado bajo todas las condiciones de mantenimiento testadas, incluso en condiciones de severa restricción nutricional en las que los individuos F no se caracterizan por presentar mayores tasas de aclaramiento (al menos inicialmente) sino un metabolismo estándar más reducido. Estos resultados indican que la branquia es un órgano

sumamente relevante en la determinación de diferencias interindividuales en tasa de crecimiento de los mejillones.

8- El análisis del transcriptoma en las branquias de individuos de crecimiento rápido y lento segregados bajo condiciones de inmersión continua y alimentados tanto con dietas de calidad alta como baja ha evidenciado la existencia de diferencias muy importantes en la expresión génica del tejido branquial entre individuos F y S. Mientras que la comparación entre individuos mantenidos en las dos condiciones de alimentación (dietas por encima o debajo del umbral de formación de pseudoheces) sólo mostro la existencia de expresión diferencial de 12 genes, en la comparación entre individuos F y S se hallaron 117 genes diferencialmente expresados. La comparación de la expresión diferencial tanto mediante el uso de términos GO (Genetic Ontology) y KEGG (Kioto Enciclopedia of Genes and Genomes) como de anotación de genes diferencialmente expresados muestra que las branquias de los individuos F sobre-expresan genes de enzimas implicadas en funciones tales como respuesta a los estímulos, crecimiento y proliferación celular (*Miostatina*, factores de crecimiento *ILGF* y *EGF*), actividad y movilidad celular (*Dynein*, *Tilb homolog protein*, *fibrocystins*), intercambio de oxígeno (*anhidrasa carbónica*), metabolismo aerobio (*Citrato Sintasa*), metabolismo de carbohidratos, estructura y funcionalidad del tejido conectivo (*Collagen*, *Laminin*, *Fibulin*, *Decorin*) y actividad filtradora (*Mucin*). En contraposición, los individuos S sobre-expresan genes implicados en las respuestas inmunológicas (*Metaloendopeptidasas*, *Galectin*, *Countin-1*), respuesta al stress (*HSP24*, *Leucine Rich Repeat protein*), metabolismo anaeróbico (*C4-dicarboxilate transporter*) y metabolismo proteico (*Pyridoxal-5 phosphate*). Las diferencias en expresión génica halladas en la branquia de individuos F y S nos permiten sugerir que la variabilidad inter-individual en el crecimiento podría deberse a las diferencias inter-individuales en el grado de inversión energética requerido para la síntesis y mantenimiento de las funciones del sistema inmunológico de defensa.